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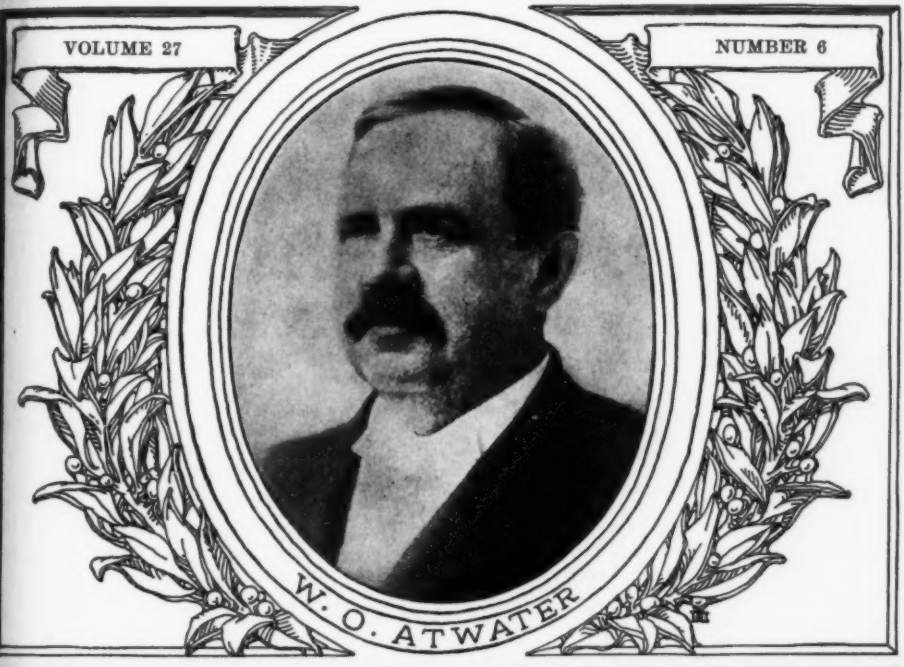

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THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPHIA 4, PA.

A STUDY OF THE RIBOFLAVIN AND THIAMINE REQUIREMENTS OF CHILDREN OF PRESCHOOL AGE

HELEN OLDHAM, FRANCES JOHNSTON, SARAH KLEIGER AND HERNAN HEDDERICH-ARISMENDI[1]

Department of Pediatrics, University of Chicago, Chicago, Illinois

TWO FIGURES

(Received for publication December 20, 1943)

In this study, the riboflavin and thiamine excretions of two normal 5-year-old boys were followed as the intakes of these vitamins were increased from very low to much higher levels. In addition, 1-hour fasting excretions, 4-hour and 24-hour returns of test doses of both vitamins, and the thiamine content of the blood were determined during the last period on each level of intake. It was hoped that this type of study would furnish information concerning the needs of children of this age for these vitamins as well as a comparison of the different methods for determining nutritional status with regard to riboflavin and thiamine.

METHODS OF STUDY

The subjects were two normal 5-year-old boys whose weights were 16.6 and 17.7 kg. They had been living in an institution for 3 months previous to this study and were known to have received good diets. During the period of study, they lived in the hospital but were given an unusual amount of freedom. When weather permitted, they were taken outside for play both morning and afternoon; otherwise, they played in a large, airy playroom. This schedule was considered comparable to that of the average city child of this age.

The experiment ran continuously for thirty-six 3-day periods with the exception of a 6-day period on one child. This interruption was due to an acute upper respiratory infection, coupled with a gastro-intestinal disorder; the study was resumed when he was apparently normal, as judged by a physician. Later in the study, both children had a similar disorder which also lasted for 6 days. During this time, urine collec-

[1] Quaker Oats Fellow of the American Academy of Pediatrics.

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tions were made as usual, although it was necessary to change their diets. The riboflavin and thiamine intakes, however, were maintained by supplements of the pure vitamins.

Diets were given which were adequate except for their riboflavin and thiamine contents. The meal pattern used throughout the study was: Breakfast — apple juice or orange juice, oatmeal or unenriched farina,[2] toast, butter and jelly; Dinner — chicken, lamb or beef, rice or potato, vegetable, fruit, bread, crackers and butter; Supper — chicken, lamb, beef, peanut butter or egg, vegetable, fruit, bread and butter, jello, ice or grape juice, cookie. Only canned vegetables and fruits were used. Those included were carrots, beets, peas, tomatoes, green beans, pears, peaches, apple sauce and pineapple. The bread was made by one of us from unenriched flour, low vitamin yeast and without milk. At each level of intake, foods which furnished the desired amounts of riboflavin and thiamine, were selected from this list and arranged in three daily menus which were used in rotation as long as the subjects were on that particular level. Milk in amounts ranging from 140 to 630 gm. per day was given in all periods except those of lowest riboflavin and thiamine intakes. In addition, the diet was supplemented with 500 mg. calcium as dicalcium phosphate; 25 mg. of ascorbic acid; 6 mg. of iron as ferric pyrophosphate and 2 teaspoonfuls of cod liver oil daily until such time as adequacy in these nutrients was assured by dietary means. Servings of foods furnishing appreciable amounts of vitamins were kept constant but the consumption of such foods as bread, butter, jelly, crackers and unenriched farina was not limited.

At the end of the study the regular hospital diet, estimated to contain approximately 800 μg. of thiamine and 1500 μg. of riboflavin, was given ad libitum for 5 days with daily supplements of 6 mg. of riboflavin and 4 mg. of thiamine.[3] Following this, the supplements were discontinued for a 2-day period before the final test doses were given.

All meals were prepared, weighed and served by one of us. At the same time samples of each food were taken for analysis. Composites representing one-fifth of the food eaten during each 3-day period, with the exception of cereals, butter and sugar, were made at the end of the period, homogenized and adjusted to approximately pH 1 by the addition of 8 ml. of concentrated hydrochloric acid per liter of suspension. Cereals were treated separately in order to rule out any untoward effect on the riboflavin assays.

[2] Kindly supplied by the Quaker Oats Company.

[3] The riboflavin and thiamine were generously supplied by Merck and Company.

Continuous urine collections were made. Each sample was immediately preserved with toluene and acetic acid and then refrigerated. One hour specimens of urine voided before breakfast were collected on 2 or more days in the periods previous to changes in the diet and analyzed separately. Riboflavin and thiamine test doses of 75 μg. per kilogram of body weight were given orally before each increase in vitamin intake. This amount was selected because it seemed to be one which could be used for children of all ages and weights without making the test doses excessively high in the case of large children and too low in the case of small ones. The thiamine and riboflavin test doses were given on successive days with vitamin low breakfasts. Consecutive 4-hour and 20-hour collections were made and analyzed separately. The 4-hour test dose return was considered to be the total excretion during the 4 hours immediately following the administration of the test dose; the 24-hour return was calculated by subtracting the average daily excretion of the previous period from the total excretion during the 24 hours following the test dose administration.

The riboflavin content of food composites and of urine was determined by the microbiological method (Snell and Strong, '39; Strong and Carpenter, '42; Silber and Unna, '42). A slightly different basal medium in which [4] the amounts of peptone and glucose were double those used in standard basal medium, was used for cereal assays. Isbell, Wooley and Fraser ('41) reported that urea in amounts greater than 20 mg. per tube inhibited the growth of lacto-bacillus casei and caused a downward drift in the riboflavin values as the amount of urine per tube was increased. We encountered no such drifts in our assays when the urine was increased from 1 to 5 ml. per tube and interpreted this as evidence that no inhibition was taking place. Urea determinations were not made but on the basis of the protein intake, the amount of urea in the tube containing 5 ml. of urine was calculated to be 40 mg. or more.

Thiamine determinations were made by the microfermentation method [5] (Atkin, Schultz and Frey, '39) as described by Knott, Kleiger and Torres-Bracamonte ('43).

[4] Suggested by Dr. Harris Isbell of the U. S. Public Health Service.

[5] After this paper was submitted for publication, an article by H. F. Deutsch ('44) appeared in which he stated that the yeast microfermentation method may give only approximate values for thiamine since other factors may also have a stimulatory effect, particularly during the pyrimidine determination. According to this, our thiamine values should be low. However, when compared with thiochrome assays on the same samples, the values obtained by the microfermentation method were usually slightly higher.

RESULTS AND COMMENT

Riboflavin

One-hour fasting excretions. After 12 to 18 days on average intakes of 250 and 275 μg. the 1-hour fasting excretions of both subjects contained only very small amounts (1–2 μg.) of riboflavin (table 1, fig. 1). Such small excretions, both relatively and absolutely, have been interpreted as an indication of near depletion (Najjar, '42). On higher intakes the excretions increased and paralleled the intakes with the exception of the periods following the upper respiratory infections. Such a gradual rise made this test of little value in studying riboflavin needs, since as yet, the amount indicative of a good nutritional state is not known. However, its eventual use as a test of nutritional status with respect to riboflavin after standards have been established seems promising, since it showed good correlation (0.83)[6] with the dietary intake.

Test dose returns. A comparison of the 4- and 24-hour returns of test doses of 75 μg. per kilogram (table 2) shows that both rose gradually as the riboflavin intake was increased and that both reflected the depleting effect of the upper respiratory infections. The correlation between the 4- and 24-hour returns was 0.89 and both showed good correlations with the dietary intake (0.76 and 0.79, respectively) and with the fasting 1-hour excretions (0.92 and 0.85, respectively). Thus it seems that the 1-hour fasting excretion, the 4-hour and the 24-hour return of a test dose all reflect equally well the nutritional status with respect to riboflavin. However, since neither the 1-hour fasting excretion nor the 4-hour return of the test dose requires the determination of the excretion during a control day on the same diet, these tests would be preferable in most cases.

Daily excretions. The average daily excretions of both subjects decreased progressively at the 250- and 275 μg. levels of intake and increased progressively at the 550-μg. level (table 1, fig. 1); this was interpreted as evidence of a gradual depletion of tissue riboflavin at the lower and a gradual rebuilding of normal stores at the higher level. If the 550-μg. intake had been continued for a longer period it is possible that eventually it might have supported even higher excretions.

[6] Correlation coefficients were calculated according to the Product-Moment formula:

$$r_{xy} = \frac{\frac{\epsilon xy}{n} - \bar{x}\,\bar{y}}{\sqrt{\left(\frac{\epsilon x^2}{n} - \bar{x}^2\right)\left(\frac{\epsilon y^2}{n} - \bar{y}^2\right)}}$$

TABLE 1

Riboflavin and thiamine excretions and thiamine blood levels on different intakes

SUBJECT 1

PERIODS	AV. DAILY INTAKE RIBOFLAVIN			RIBOFLAVIN EXCRETION			PERIODS	AVERAGE DAILY INTAKE OF THIAMINE		THIAMINE EXCRETION			BLOOD THIAMINE PER 100 ML.
	Calories	Calculated [1]	Determined	Av. per 24 hr. in each period	4-hr. after test dose	Av. per 1 hr. fasting [2]		Calculated [1]	Determined	Av. per 24 hr. in each period	4-hr. after test dose	Av. per 1 hr. fasting [2]	
		μg.	μg.	μg.	μg.	μg.		μg.	μg.	μg.	μg.	μg.	μg.
1-6	1290	300	250	50, 40, 30, 20, 20, 20	140	1	1–6	225	150	90, 20, 10, 20, 10, 30	30	3	4.9
7-12	1390	700	550	30, 40, 40, 40, 70, 70	160	4	7–16	475	475	50, 50, 40, 90, 90, 60, 40, 60, 50, 50	20	2	6.9
13-18	1310	900	700	130, 140, 130, 140, 110, 50	240	9	17–20	575	600	130, 70, 120, 110	150	3	6.3
19-26 [3]	1450	1150	850	100, 70, 470, 200 180, 180, 120, 90	100	3	21–26 [3]	750	700	150, 90, 190, 170, 150, 230	..	..	..
27-31	1370	1375	1050	90, 230, 230, 200, 210	110	13	27–31	775	700	170, 130, 100, 180, 210	150	6	8.4
32-36	1540	1850	1450	320, 340, 330, 400, 370	420	23	32–36	900	950	280, 290, 280, 370, 280	160	11	7.5
37	....	7500	....	5080	...	..	37	4800	...	2370	...	..	..
38	....	1500	....	990	440	35	38	800	...	950	300	17	8.4
SUBJECT 2													
1-4	1520	325	275	50, 20, 20, 30	80	2	1–4	250	200	120, 30, 30, 50	70	2	3.6
5-10	1570	725	550	30, 30, 50, 60, 70, 80	150	5	5–14	500	525	40, 100, 100, 150, 90, 90, 90, 80, 60, 60	80	3	4.8
11-16	1380	900	725	110, 90, 100, 90, 100, 140	220	9	15–20	575	600	50, 130, 140, 130, 160, 120	130	4	5.3
17-26 [3]	1410	1125	875	170, 160, 210, 150, 340, 440, 300, 170, 100, 110	140	7	21–26 [3]	750	700	180, 180, 220, 180, 230, 120	...	..	..
27-31	1690	1425	1075	160, 140, 220, 250, 270	280	14	27–31	825	750	160, 180, 140, 150, 130	160	7	7.1
32-36	1520	1775	1425	470, 430 320, 490, 440	360	23	32–36	875	925	290, 310, 220, 280, 260	180	7	7.7
37	....	7500	....	5050	...	..	37	4800	...	1560	...	..	..
38		1500	...	830	440	25	38	800	...	800	300	11	6.5

[1] Both the riboflavin and the thiamine content of the diet were calculated from a table of values which was compiled after a survey of published data from many sources. Recipes were calculated and losses in cooking and canning were taken into consideration. These calculations were made for comparison with the determined values on the composites. The discrepancy between calculated and determined riboflavin values is due chiefly to the riboflavin content of the milk which was calculated as 200 μg. per 100 gm. Analyses showed it to contain approximately 180 μg. per 100 gm.

[2] The 1-hour fasting values represent the average of two or more determinations, carried out on successive days.

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		μg.	μg.	μg.	μg.	μg.		μg.	μg.	μg.	μg.	μg.	μg.
SUBJECT 1													
1–6	1290	300	250	50, 40, 30, 20, 20, 20	140	1	1–6	225	150	90, 20, 10, 20, 10, 30	30	3	4.9
7–12	1390	700	550	30, 40, 40, 40, 70, 70	160	4	7–16	475	475	50, 50, 40, 90, 90, 60, 40, 60, 50, 50	20	2	6.9
13–18	1310	900	700	130, 140, 130, 140, 110, 50	240	9	17–20	575	600	130, 70, 120, 110	150	3	6.3
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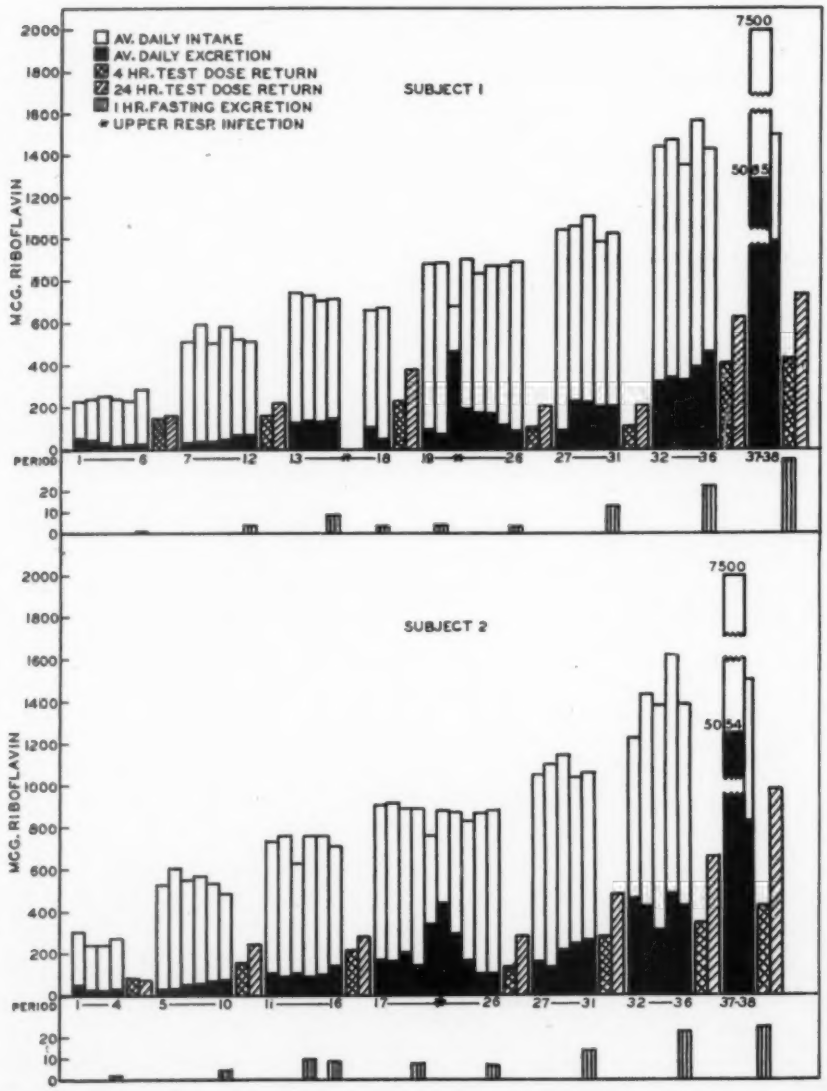

Fig. 1 Average daily excretions, test dose returns and fasting excretions of riboflavin at different intake levels.

The comparatively constant excretions, however, on the 700- and 725-μg. intakes (0.53 mg. per 1,000 Cal.), made it seem quite certain that this amount met the requirements of these subjects, and would therefore be an adequate allowance for children of this age. When the intake was at this level, the average daily excretions were 117 and 105 μg.; the 1-hour fasting excretions were 9 μg.; the 4-hour returns of test doses were 20 and 17% and the 24-hour returns of test doses were 32 and 23%.

TABLE 2

Comparison of 4- and 24-hour returns of 75 μg. per kilogram test doses[1] of riboflavin and thiamine

SUBJECT	RIBOFLAVIN			THIAMINE		
	Average dietary intake	Test dose return		Average dietary intake	Test dose return	
		4 hr.[2]	24 hr.[3]		4 hr.[2]	24 hr.[3]
	μg.	%	%	μg.	%	%
1	250	11	13	150	2	13
2	275	6	6	200	5	9
1	550	13	18	475	1	5
2	550	12	19	525	6	12
1	700	20	32	600	13	22
2	725	17	23	600	10	32
1	850	9	17	700	..	..
2	875	11	22	700	..	..
1	1050	8	17	700	12	17
2	1075	20	36	750	12	18
1	1450	33	50	950	13	22
2	1425	26	47	925	13	29
1	1500[4]	35	59	800[4]	23	57
2	1500[4]	32	71	800[4]	22	55

[1] Test doses for subject 1 were approximately 1250 μg.; for subject 2, 1350 μg.

[2] Total excretion during 4 hours following test dose administration with a vitamin low breakfast.

[3] Total excretion during 24 hours following test dose administration less the average daily excretion of the previous period.

[4] Calculated intakes following the 5 days when supplementary vitamins were given.

The extremely high excretions of riboflavin of subject 1 during period 21 and of subject 2 during periods 21, 22 and 23 were obviously due to the acute upper respiratory infections with accompanying elevations of body temperature which probably caused a coincident lack of protein deposition. This would be in agreement with the findings of Sarett et al. ('42, '43) that the riboflavin excretion of dogs and rats varied inversely with the protein intake and that protein deposition was necessary for riboflavin retention in the liver. During subsequent periods in

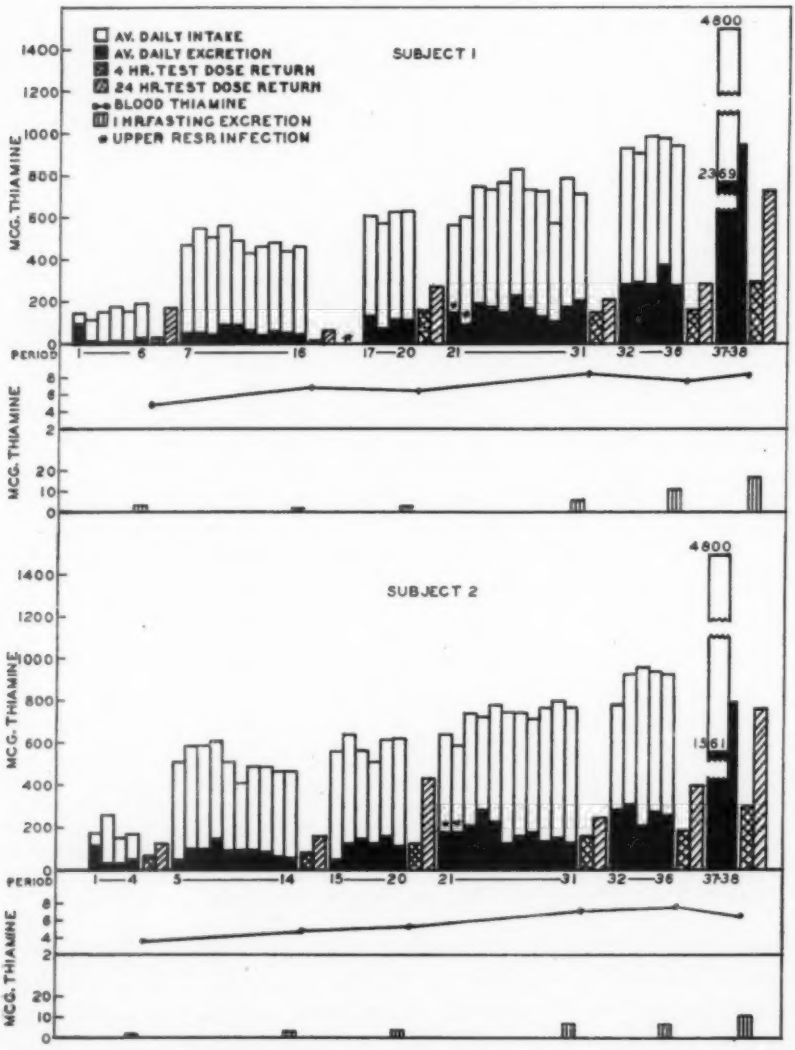

Fig. 2 Average daily excretions of thiamine, test dose returns, fasting excretions and blood thiamine at different intake levels.

which both children were apparently normal in every respect and when compensatory protein deposition might have been expected the riboflavin excretions were low. This effect was also noted after a similar illness of subject 1 which occurred following period 16. Collections were discontinued during the time of the illness but his excretion of riboflavin was lower in periods 17–20 than it had previously been on a similar intake. These lowered excretions continued for 12 to 15 days after the children had apparently recovered which suggests the need for increased riboflavin intake after this type of illness.

Average daily excretions of over 300 μg. on 1450- and 1425-μg. intakes (periods 32 to 36) and of over 5000 μg. on a 7500-μg. intake in period 37 made us believe that the tissues of these subjects were saturated with riboflavin on the 1450- and 1425-μg. intakes. This viewpoint was further substantiated by the similarity of the test dose returns on the two levels of intake.

Riboflavin requirement. A riboflavin intake of approximately 0.50 mg. per 1,000 Cal., and amount one-third less than the 0.75 mg. per 1,000 Cal. provided by the dietary allowances of the Food and Nutrition Board of the National Research Council, seemed to be ample for the subjects of this study. At this level of intake (0.53 mg. per 1,000 Cal.) the fasting 1-hour excretions were 9 μg., and 4-hour test dose returns approximately 20%. Excretions of this magnitude, therefore, might be considered indicative of satisfactory nutritional status with respect to riboflavin until more data are available.

Thiamine

One-hour fasting excretions. The 1-hour fasting thiamine excretions followed the same general pattern as those of riboflavin (table 1, fig. 1). They were from 2 to 4 μg. on intakes of less than 700 μg. but increased to 6 and 7 μg. at that level. Like the riboflavin excretions, they correlated well (correlation coefficient 0.85) with the dietary intake; this made it seem that this test reflected the nutritional status with respect to thiamine.

Test dose returns. The returns of thiamine test doses of 75 μg. per kilogram (table 2) were small on the low thiamine intakes but both the 4- and 24-hour returns showed marked increases at the 600-μg. level. No further increases of any magnitude occurred until after the subjects had received supplementary thiamine in period 37. No evidence of the depleting effects of the upper respiratory infections was shown, as was the case with respect to the riboflavin test dose returns. This, how-

ever, was not surprising, since the daily excretions of thiamine during periods 21 and 22 were not abnormally high.

The correlation between the 4-hour and the 24-hour returns was 0.91 and both showed good correlations (0.86 and 0.82, respectively) with the fasting 1-hour excretions as well as with the intakes (0.87 and 0.92, respectively). Again, as in the case of riboflavin the decision as to which of these three tests to use would depend on the particular situation rather than the superiority of any one of them.

Blood thiamine. The thiamine blood levels were slightly lower than 5.0 μg. per 100 ml. of whole blood on the 150- and 200-μg. intake levels. This figure was suggested as optimal for infants by Knott ('42). However, since Benson et al. ('42) found an average blood level of 7.8 μg. per 100 ml. in a group of forty-five normal children and since we found that the average blood thiamine of a group of thirty-nine normal children was increased from 5.5 to 7.3 μg. per 100 ml. when the diet was improved,[1] it is possible that for good nutrition a level of approximately 7.0 μg. per 100 ml. is desirable. The two subjects in this study had values for blood thiamine of this magnitude on intakes of 700 and 750 μg., respectively.

The correlation coefficients of the blood thiamine with the fasting 1-hour excretions, 4-hour and 24-hour returns of test doses and average intakes were 0.66, 0.58, 0.40 and 0.45, respectively. Therefore, it seems as though less confidence can be placed in this test than in the excretion tests as a measure of nutritional status.

Daily excretions. The average daily excretions of thiamine adjusted to a changed intake quite rapidly (fig. 1) after which they remained fairly constant at all levels of intake. The per cent of ingested thiamine excreted increased gradually with the intake and when the latter reached 600 μg., 18% was excreted by subject 1 and 20% by subject 2. On intakes of 700 and 750 μg., the per cent excreted was only slightly higher (23%) but on the next level studied (950 and 925 μg.), it increased to 30%. When the intakes were increased to 4800 μg., 49% was excreted by one subject and 33% by the other.

Thiamine requirements. If an excretion of 20% or more of the daily thiamine intake can be considered indicative of adequacy, as suggested by Benson et al. ('42), it would seem that 600 μg., satisfied the needs of these subjects fairly well. The sudden increase in test dose returns at this level, gives it further support. On the other hand, at the 700- to 750-μg., intake level, the per cent of dietary thiamine excreted daily was not much above that at the 600-μg.; the 1-hour fasting excretions showed

[1] Unpublished data.

their first significant increase at this level of intake and the thiamine blood levels were higher. This indicated that 750 μg., of thiamine (approximately 0.50 mg. per 1,000 Cal.) would probably be a safer allowance for children of this age until further data are available. This amount was that recommended by the Food and Nutrition Board of the National Research Council for children of this age. It also agrees fairly well with the 0.45 mg. per 1,000 Cal. suggested by Benson et al. ('42a) for children and by Williams et al. ('43) as the minimum requirement of the adult. At this level, the daily excretions were 23% of the intake; the fasting 1-hour excretions were 6 and 7 μg.; the blood thiamine levels were 8.4 and 7.1 μg. %; the 4-hour test dose returns were 12% and the 24-hour test dose returns 17 and 18% of the test dose.

SUMMARY AND CONCLUSIONS

The average daily urinary excretions of riboflavin and thiamine of two preschool children were determined on various levels of intake. One-hour fasting excretions, 4- and 24-hour returns of test doses of both vitamins and thiamine content of the blood were determined at each intake level.

The 1-hour fasting excretions and the 4- and 24-hour test dose returns were found to correlate well with each other and with the intake. There was less evidence of correlation of blood thiamine with both the intake and the other tests of nutritional status. It seems, therefore, that the 1-hour fasting excretion and the return of an oral test dose can be used equally well in determining the nutritional status with respect to riboflavin and thiamine but that the blood thiamine level is less reliable.

The riboflavin requirement was considered to be the lowest level at which the daily excretions neither decreased nor increased progressively. In the case of thiamine, the requirement was based on an average daily excretion of 20% of the intake, decided increases in the fasting 1-hour excretions and test dose returns, and blood levels of 7.0 μg. or more per 100 ml. Riboflavin and thiamine intakes of approximately 0.50 mg. per 1,000 Cal. satisfied these criteria and therefore appeared to meet the needs of the subjects used in this study.

Until such time as more data are available, the return, in 4 hours, of 20% of a test dose of riboflavin and of 12% of a test dose of thiamine or fasting 1-hour excretions of 9 μg. of riboflavin and 6 μg. of thiamine might be considered indicative of satisfactory nutritional status with respect to these vitamins.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Miss Marjorie Young and Miss Grace Herzog, who assisted in this study.

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VITAMIN INTERRELATIONSHIPS

III. INFLUENCE OF SUB-OPTIMUM DOSES OF THIAMINE ON URINARY EXCRETIONS OF RIBOFLAVIN [1]

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Recently Sure and Ford ('42) reported that in thiamine deficiency there is a pronounced disturbance in riboflavin metabolism. Such observations were made on animals that were thoroughly depleted of thiamine, hence losses of weight were encountered in some of the avitaminotic animals, although in some groups the normal animals restricted in food to that consumed by the pathological litter mates actually lost more weight than the vitamin B_1 deficient rats. These weight changes, not given in the paper referred to, were taken in consideration in arriving at the conclusion drawn that the marked disturbance in riboflavin metabolism produced by thiamine deficiency is due mainly to poor retention and not necessarily to body tissue catabolism.

Since border-line rather than marked thiamine deficiencies are more commonly found in this country, it was thought of interest to investigate the influence of chronic thiamine deficiency on riboflavin utilization. Since urinary excretions gave an index of retention and fecal riboflavin excretions did not contribute any material information to the picture in our previous study (Sure and Ford, '42), this investigation was limited to only urinary excretions. Previously we found large individual differences in fecal excretions of riboflavin and it was, therefore, felt an additional set of data would not appreciably influence the relative results on retention, if noteworthy changes were observed in urinary excretions of riboflavin on different doses of thiamine.

The experiments were carried out in four series, the results of which are summarized in table 1.

[1] Research paper no. 784 Journal series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station. This investigation was aided by a grant from the Williams-Waterman Fund of the Research Corporation.

TABLE 1

Influence of sub-optimum thiamine intake on urinary excretions of riboflavin

SERIES	RATION	METABOLISM PERIOD	EXPERIMENTAL PERIOD	NO. OF ANIMALS	DAILY THIAMINE INTAKE	TOTAL RIBOFLAVIN INTAKE	CHANGES IN BODY WEIGHT	RIBOFLAVIN EXCRETED IN URINE[1]	PER CENT OF RIBOFLAVIN INTAKE EXCRETED IN URINE
		days	*days*		*μg.*	*μg.*	*gm.*	*μg.*	
A	5357	7	58th to 65th	4	1 to 2	140	— 13.8	43.8	31.3
A	5357	7	58th to 65th	4	3	140	— 4.5	29.4	21.0
A	5357	7	58th to 65th	4	4	140	— 4.2	33.3	23.1
A	5357	7	58th to 65th	4	5	140	— 3.5	29.0	20.7
A	5357	7	58th to 65th	4	6	140	— 3.3	27.6	19.7
A	5357	7	58th to 65th	4	11	140	— 0.3	25.4	18.1
A	5357	7	71st to 78th	4	2 to 4[2]	70	— 2.0	33.7	48.1
A	5357	7	71st to 78th	4	3	70	— 1.3	18.4	26.3
A	5357	7	71st to 78th	4	4	70	— 2.0	16.2	23.1
A	5357	7	71st to 78th	4	5	70	— 1.0	14.9	21.3
A	5357	7	71st to 78th	4	6	70	— 1.3	11.9	17.0
A	5357	7	71st to 78th	4	11	70	+ 1.0	13.9	19.8
A	5388	13	80th to 93rd	4	4	130	+ 6.5	34.5	24.7
A	5388	13	80th to 93rd	4	8	130	+ 7.5	19.6	15.2
A	5388	13	80th to 93rd	4	12	130	+ 8.3	11.8	9.1
A	5388	13	80th to 93rd	4	16	130	+ 7.3	8.4	6.5
A	5388	13	80th to 93rd	4	20	130	+ 7.8	8.8	6.7
A	5388	13	80th to 93rd	4	50	130	+ 8.5	16.9	13.0
B	5388	13	180th to 193rd	4	1 to 3	260	— 7.3	95.9	36.9
B	5388	13	180th to 193rd	4	4	260	+ 2.7	52.3	20.1
B	5388	13	180th to 193rd	4	6	260	+ 3.0	23.3	8.9
B	5388	13	180th to 193rd	4	8	260	— 0.3	25.2	9.7
B	5388	13	180th to 193rd	4	10	260	+ 1.3	20.3	7.8
B	5388	13	180th to 193rd	4	20	260	+ 2.8	22.0	8.5
B	5388	9	195th to 204th	4	1 to 3	90	— 2.3	31.8	35.3
B	5388	9	195th to 204th	4	4	90	— 0.3	23.9	26.5
B	5388	9	195th to 204th	4	6	90	— 0.5	6.9	7.6
B	5388	9	195th to 204th	4	8	90	+ 1.0	6.8	7.3
B	5388	9	195th to 204th	4	10	90	± 0.0	6.5	7.2
B	5388	9	195th to 204th	4	20	90	— 1.8	3.5	3.9

[1] Corrected for the amount excreted on a riboflavin-deficient ration. The figures in this table represent averages per animal from data secured on four animals.

[2] Animals were in depleted thiamine state and it was necessary to administer a minimum of 2 to 4 μg. of this vitamin daily to prevent polyneuritis and insure adequate food consumption to circumvent rapid catabolism.

MODIFIED PROCEDURE FOR DETERMINATION OF RIBOFLAVIN IN RAT'S URINE

Before presenting the results of this investigation a brief description should be given of the modified analytical procedure used for the determination of riboflavin in the urine. The urine is collected in amber-colored bottles containing 10 to 12 ml. N/5 H_2SO_4 to which has been added 15 drops of toluene. The urine is analyzed four times weekly. During 3 days of the week the urine, adjusted to a pH of about 1 and preserved with toluol, is kept in a refrigerator. In the mornings the funnels are washed with a fine spray of enough distilled water to allow 200 ml. dilution for a 48-hour sample and a 100 ml. dilution for a 24-hour sample. Ten ml. aliquots are transferred into 50-ml. amber-colored Erlenmeyer flasks. The pH is adjusted to about 4.5 with 2N sodium acetate buffer, using bromo-cresol-green as an outside indicator. One drop of a saturated solution of $KMnO_4$ is added, the flasks are rotated gently several times during 2 minutes, which is sufficient time for the oxidation of foreign fluorescent substances to take place. One drop (and only occasionally 2 drops) of a 3% solution of H_2O_2 removes the excess of $KMnO_4$ and the diluted urine solution clears up without foaming. Five ml. of distilled water are now added, so that the cuvette for the Pfaltz and Bauer fluorophotometer contains exactly 15 ml. total volume. The instrument is set so that the galvanometer reads 25 for 1 μg. riboflavin. The whole operation is so rapid that we generally run twelve determinations consecutively. The riboflavin is then reduced to the colorless form with 10 to 20 mg. pure sodium hydrosulfite crystals. The latter reagent need not be weighed but can be roughly estimated by the amount that can be picked up on the tip of a spatula. The riboflavin in the urine is computed from a calibrated curve which is linear. All riboflavin determinations are carried out with the curtains of the laboratory drawn, so as to exclude as much light as possible.

We find the procedure described above much more accurate and rapid than the one used previously (Sure and Ford, '42). The two main advantages are that it prevents foaming of the diluted urine sample, and the decomposition of the aqueous solution of sodium hydrosulfite, used previously, during the determinations is circumvented.

SERIES A EXPERIMENTS

In this series there were six male and eighteen female rats. They were started on experiments when they were 35 days of age and weighed 47 to 65 gm. each. The investigation was carried out on twenty-four animals in four groups of six rats in each group. The animals were

[1] Animals were in depleted thiamine state and it was necessary to rid them of polyneuritis and insure adequate food consumption to circumvent rapid catabolism.

given ration 5357 of the following percentage composition: casein (vitamin-free)[2] 18; agar-agar, 2; salts no. 1 (Sure, '41), 4; butter fat, 10; polished rice, 30; and cerelose, 36. Thiamine was administered daily in graduated doses, and according to the set up, four animals were available for each dose. The first animal received the smallest dose and the amount of feed it consumed was allotted to the rest of the five animals in each group. The thiamine daily doses were 0, 2, 3, 4, 5, and 10 μg., respectively. After 4 weeks the first animal was given 1 μg. B_1 daily. To these doses one must add the 1 μg. contributed daily by the polished rice in the ration. Other vitamin supplements were given daily as follows: 20 μg. riboflavin, 20 pyridoxine, 6 mg. choline chloride and 100 μg. calcium pantothenate, later increased to 200 μg. As a source of vitamins A and D, 3 drops of halibut liver oil were given once weekly to each animal. The same vitamin supplements were administered to the rest of the animals in series B following. Since the animals in series A were on isocaloric diets, they were equalized with respect to weights during the three metabolism periods, the weights ranging from 110 to 120 gm. each.

The first 7-day metabolism study was carried out on the fifty-eighth to sixty-fifth days of the experiment. From table 1 it is apparent that only the animals which received the 1 to 2 μg. thiamine daily showed a greater excretion of riboflavin in the urine. However, since the rats which received these thiamine doses lost more weight than the rest of the animals in this group, the greater excretion of riboflavin may be attributed to losses from the tissues produced by body catabolism. This picture was revealed from consideration of the effect of the 20 μg. daily dose of riboflavin. However, during the next 7-day metabolism period, when the daily dose of riboflavin was reduced to 10 μg., 6 to 11 μg. of thiamine produced the lowest excretion of riboflavin in the urine (table 1). The animals which previously received 1 to 2 μg. daily were in an advanced avitaminotic state and had to be given 3 to 4 μg. daily to prevent polyneuritis and marked anorexia, and because of that depleted state, were excreting more riboflavin in the urine than the litter mate controls, which were receiving 3 μg. thiamine daily. The weight changes during the second as well as third metabolism period were negligible, which eliminates body tissue catabolism as a factor in the interpretation of the results. Further evidence of a thiamine-riboflavin interrelationship in chronic B_1 deficiency became evident in the third metabolism period of 10 days when this group of animals was given purified synthetic diet 5388, which is the same as ration 5357,

[2] SMACO.

modified by replacement of 30% polished rice with an equivalent amount of cerelose. It will be noted that on this purified diet free from natural foods the optimum thiamine dose for 10 μg. riboflavin is 12 to 16 μg. Increasing the thiamine dose from 4 to 16 μg. decreased the riboflavin excretion in the urine, calculated as per cent of total intake, from 24.7 to 6.5%. In other words, on the 16 μg. daily dose of thiamine a little less than ¼ of the riboflavin intake was excreted in the urine as on the 4 μg. daily dose of thiamine. However, the increase in urinary excretion of riboflavin when the daily thiamine was increased from 20 to 50 μg. was not confirmed by later experiments.

SERIES B EXPERIMENTS

This series of experiments was conducted with twelve male and twelve female rats. They were started when 35 days old, weighing 42 to 68 gm. each. The animals were placed on ration 5388 and during the first 111 days feed was allowed ad libitum, and the daily thiamine doses were 0 to 2, 2, 3, 4, 5, and 15 μg., respectively. The experiments were carried out as in series A, with four groups of animals, six in each group. From the one hundred and eleventh day on isocaloric diets were fed and the daily thiamine doses were changed to 1 to 3, 4, 6, 8, 10, and 20 μg. In spite of differences in weight precipitated by the ad libitum feeding the first 111 days of the entire 204 days of the experimental period, the chronic avitaminosis produced by administration of inadequate amounts of thiamine, was responsible for large excretions of riboflavin in the urine on daily thiamine doses below 6 μg. (table 1). This occurred when the daily dose of riboflavin was either 10 or 20 μg.

In the first metabolism period, on the 20 μg. daily dose of riboflavin the increase of the daily intake of thiamine from 1–3 to 10 μg. reduced the urinary excretion of riboflavin, expressed as per cent of intake, from 36.9 to 7.8, or about one-fifth. Similarly, in the second metabolism experiment, when the daily riboflavin dose was 10 μg., the increase in the daily intake of thiamine from 1–3 to 6 μg. reduced the urinary excretion of riboflavin from 31.8 to 6.9% of the total intake. It is thus evident that in chronic thiamine deficiency, uncomplicated by body tissue catabolism, greater excretions of riboflavin occur on sub-optimum intakes of vitamin B_1. Such greater excretions of riboflavin, as shown in a recent publication (Sure and Ford, '42) represent relatively lesser retention of this vitamin; hence it follows that on sub-optimum doses of thiamine which produce a chronic B_1 avitaminosis, there is a poor retention of riboflavin.

DISCUSSION

It is apparent from the results of the experiments submitted in this communication that a definite thiamine-riboflavin interrelationship exists in chronic as well as in acute thiamine deficiency (Sure and Ford, '42). During 2 months of inadequate thiamine intake no significant changes were observed in urinary excretions of riboflavin on a 20-μg. daily dose of this vitamin, but great excretions of riboflavin in the urine occurred on a 10-μg. daily dose. However, on a 6-month sub-optimum thiamine intake large excretions of urinary riboflavin occurred on a 20- as well as a 10-μg. daily dose of riboflavin. The question arises as to the possible significance of these findings in human experience. Ferrebee and Waisman ('43) attempted to answer this question but their experiences with rats were limited to only a few weeks thiamine depletion and they were dealing with acute and not with chronic thiamine avitaminosis. Also, the clinical results submitted by these investigators on four patients could hardly be considered as convincing, particularly since these patients, although suffering from malnutrition, were not representative chronic or advanced cases of thiamine deficiency. It is suggested that, if any relationship between chronic thiamine deficiency and riboflavin retention exists in the human, evidence should be obtained from numerous cases of chronic border-line deficiencies in anorexias of children, polyneuritis of pregnancy, alcholic polyneuritis, and in patients suffering from cardiovascular dysfunction associated with B_1 avitaminosis (Weiss and Wilkins, '36 and '37; and Weiss, '40).

SUMMARY

Chronic thiamine deficiency produces great losses of riboflavin in urine, uncomplicated by body tissue catabolism, and hence lowers the retention of the latter vitamin.

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ASSOCIATIVE DYNAMIC EFFECTS OF PROTEIN, CARBOHYDRATE AND FAT[1]

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TWO FIGURES

(Received for publication December 23, 1943)

It is the understanding of the authors that a nutrient fed alone is never metabolized alone, and that the apparent energy expense of utilization, or heat increment, or dynamic effect, of a nutrient is much affected (1) by the nutrient combination in which it is metabolized, (2) by the method of disposition of the absorbed nutrients by the experimental subject, and (3) by the technic of measurement of the dynamic effect as representing a single feeding of a test nutrient or a difference between higher established states of nutrition.

In this light the present investigation was conducted to determine the dynamic effects in mature, male albino rats of protein, carbohydrate and fat, singly and in four combinations, all fed as supplements to the same quantity of the same nutritively complete, basal, maintenance diet.

The advantages of the general procedure followed, as compared with the measurement of dynamic effects of single test meals fed to animals in a post-absorptive state, are that the results are more representative of nutritive practice; they are not confused with the dynamic effects of katabolized body nutrients; and they are both more significant and apt to be more accurate, since they are measured as the difference between the amounts of heat produced at established planes of metabolism, without need for concern as to the beginning, the maximum and the end of the dynamic effect as of the single test meal.

For the information of readers who are accustomed to determining dynamic effects of feeds from single portions of the test substances fed to animals in a post-absorptive state, and who therefore expect to

[1] Authorized for publication on December 18, 1943, as paper no. 1214 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

observe a marked rise and subsequent fall in heat production while the animal utilizes the test substance, the authors advise that the difference between the heat production of fasting rats and rats receiving feed is small, especially as compared with the comparable value for ruminants, and that they have a great volume of unpublished data showing that when rats are fed twice per day there is no falling off in the heat production, as indicated by the elimination of carbon dioxide, at the end of 7-hour periods of observation under the conditions of the present study. Therefore, when dynamic effects of nutrients are determined by the comparison of the heat production of the same group of rats established consecutively on two planes of nutrition above energy equilibrium, the variations in the heat production during the two periods of observation are considered to cancel out by subtraction.

A conceivable minor imperfection in the determination of dynamic effects at planes of nutrition above maintenance, however, is that they do not include the amount of energy which would be required to eliminate the nutrients retained as part of body tissue.

The literature relating to the dynamic effects of nutrients has not been reviewed in connection with this paper because it has been recently reviewed by Wilhelmj ('35), Borsook ('36), Murlin ('39), and Kriss ('41), and because there has been no previous study of the present subject by the general procedure followed.

EXPERIMENTAL

The general technic employed, which is derived from Armsby's studies of the energy metabolism of cattle, depending on the comparison of the heat production from established states of nutrition, has been used at this institute since 1902 — recently in studies of the dynamic effects of nutrients for the albino rat (Kriss et al., '34; Kriss, '38; Forbes et al., '39b), and is here utilized, with special adaptations, in the further investigation of this latter subject.

An advantage of this procedure is that conditions which affect the rate at which the diet is metabolized are without effect on the heat increment since these conditions prevail both at the beginning and the end of the period of observation.

In recognition of the considerable individual variation in the heat production of animals, twelve experimental subjects were used for each observation. The experimental program, as shown in table 1, was so arranged that periods compared were immediately contiguous. The subjects were of exactly the same age and approximately the same body weight at the beginning of each unit of the investigation. Each

comparison of heat production was made between two sets of observations on a single group of rats, but a different group of rats was used for each such comparison. Also the subjects were trained for the respiration experiments by subjection to the experimental conditions in blank runs on at least 2 days prior to the respiration measurements.

TABLE 1

Schedule of experimentation.

RAT NUMBERS	DAILY FOOD INTAKE	AGE OF RATS	AVERAGE WEIGHT OF RATS	DATES OF RESPIRATION MEASUREMENTS
		days	*gm.*	
1–12	10 gm. basal diet	170	298	Oct. 12–17
1–12	10 gm. basal + 4 gm. cerelose (corn sugar)	177	308	Oct. 19–24
13–24	10 gm. basal diet	170	282	Nov. 9–14
13–24	10 gm. basal + 3.54 gm. beef protein	177	293	Nov. 16–21
25–36	10 gm. basal diet	170	269	Dec. 7–12
25–36	10 gm. basal + 1.5 gm. lard	177	277	Dec. 14–19
37–48	10 gm. basal diet	170	286	Jan. 4–9
37–48	10 gm. basal + 2 gm. cerelose + 1.848 gm. beef protein	177	300	Jan. 11–16
49–60	10 gm. basal diet	170	297	Feb. 1–6
49–60	10 gm. basal + 2 gm. cerelose + 0.737 gm. lard	177	303	Feb. 8–13
61–72	10 gm. basal diet	170	301	Mar. 1–6
61–72	10 gm. basal + 1.848 gm. beef protein + 0.737 gm. lard	177	306	Mar. 8–13
73–84	10 gm. basal diet	170	286	Mar. 29–Apr. 3
73–84	10 gm. basal + 1.333 gm. cerelose + 1.232 gm. beef protein + 0.491 gm. lard	177	297	Apr. 5–10

The subjects were sexually mature and had passed the period of most rapid growth, but under favorable conditions were still able to retain significant proportions of their nitrogen intake, as shown in table 2.

In each unit of the investigation each of the twelve subjects was used first for individual determination of the metabolizable energy and the heat production from 10 gm. of the basal diet, followed by the same determinations with reference to this basal diet plus one of the test supplements in a quantity computed to yield, in each case, approximately the same amount of metabolizable energy. The dynamic effects of the supplements, therefore, were computed by difference between the quantities of heat produced from the basal and the supplemented diets.

The consecutive time intervals occupied by these measurements were as follows: (a) Basal diet — introductory period, 10 days; excreta

collection for determination of metabolizable energy, 8 days; respiration experiment, 2 days; (b) Supplemented diet — introductory period, 5 days; respiration experiment, 2 days; excreta collection for determination of metabolizable energy, 8 days. The special purpose of this arrangement was to provide that the measurements of the heat production on the supplemented diet should follow the measurements on the basal diet with the shortest practicable intervening interval of time.

The heat production was determined by the respiratory quotient procedure. Protein metabolism was computed from the urinary nitro-

TABLE 2

Utilization of daily nitrogen.

RAT NUMBERS	FEEDING TREATMENTS	NITROGEN INTAKE	NITROGEN DIGESTED	NITROGEN OF URINE	NITROGEN RETENTION
		mg.	%	%	%
1–12	Basal diet	365	69.0	61.7	7.3
1–12	Basal + cerelose	365	71.1	50.5	20.6
13–24	Basal diet	365	68.4	54.3	14.1
13–24	Basal + beef protein	864	82.8	63.7	19.1
25–36	Basal diet	365	68.0	69.9	— 1.9
25–36	Basal + lard	354	70.2	42.8	27.4
37–48	Basal diet	365	66.8	56.9	9.9
37–48	Basal + cerelose + beef protein	632	79.7	54.9	24.8
49–60	Basal diet	365	68.3	64.7	3.6
49–60	Basal + cerelose + lard	362	68.8	50.9	17.9
61–72	Basal diet	365	70.0	60.8	9.2
61–72	Basal + beef protein + lard	622	80.3	61.2	19.1
73–84	Basal diet	365	70.4	51.6	18.8
73–84	Basal + cerelose + beef protein + lard	543	78.4	59.3	19.1

gen. Carbon dioxide production was determined by absorption, and oxygen utilization determined by the method of Haldane as the combined weights of carbon dioxide and moisture given off minus the loss in weight of the animal.

The factors employed in this computation (Loewy and Oppenheimer, '11) were the following:

Urinary N (mg.) $\times$ .00476 = liters CO_2 from protein.
Urinary N (mg.) $\times$ .00594 = liters O_2 used by protein.
Urinary N (mg.) $\times$ 26.51 = calories from protein.

Caloric values per liter O_2, at respiratory quotients as determined, are given in Lusk's modification (Lusk, '24) of the table of Zuntz and Schumburg ('01). In those cases in which the non-protein respiratory quotient exceeded unity the caloric value of the excess CO_2 was reckoned as 1.09 Cal. per liter, as determined by Bleibtreu ('01).

The rats were fed twice a day, and heat production was measured on 2 consecutive days during 7-hour periods beginning at 8:30 A.M., about 1½ hours after the morning feeding.

The weight of the carbon dioxide absorbed, and the degree of physical activity of the experimental subject as indicated by a work adder, were recorded each hour, and the values used for heat production were selected as those representing intervals of time during which the physical activity and the heat production were at their lowest levels. The weight of carbon dioxide eliminated during the entire 7-hour period was used in computing the respiratory quotient, but only the amount eliminated during the selected intervals of quiet were used in computing the heat production. It was assumed that the total respiratory quotient was unaffected by the slight activity of the animal during the 7-hour respiration period. The definite significance of the results obtained, therefore, was limited to the selected intervals of quiet, without evidence or claim that they exactly represented the entire day of 24 hours. It is also conceded that there was probably a difference between the amounts of heat produced by the subjects while asleep and while awake but motionless. The respiration experiments were conducted with the apparatus at a temperature of 28°C. ± .1°C., the temperature inside the respiration chamber being about 29°C.

The dates of the respiration measurements, as given in table 1, cover the intervals of 6 days during which each of the twelve rats on a dietary treatment was subjected to respiration measurements on 2 consecutive days, tests being made on four rats each day.

The basal diet was a complete commercial feed manufactured for dogs; the protein supplement was dried and extracted beef muscle; the carbohydrate supplement was a corn sugar product, called cerelose; and the fat used was commercial lard.

The differences in the body weights of the rats at ages 170 and 177 days represented in part, each, gain in body substance and in alimentary fill. Since these gains in weight were small, and since the extent, in each case, to which they represented body gain and increase in alimentary fill was not in evidence, no effort was made to correct the heat production to correspond to the same body weight in the basal and the supplemented periods.

RESULTS — DISCUSSION

Referring to table 2, the nitrogen intake in the diets was essentially the same, as also was the apparent digestibility of the nitrogen, in all diets except those containing beef muscle protein. In these cases the

apparent digestibility of the nitrogen of the diets was materially increased by the added amounts of highly digestible protein. This supplementary beef protein presumably added little either to the undigested nitrogen, or to the metabolic nitrogen, of the feces.

As compared with the nutritive status of the rats on the basal diet — with any greater amount of either nitrogenous or non-nitrogenous nutriment there was an increase in the percentage of the food nitrogen digested and in the percentage retained. Also the quantity of nitrogen in the urine was increased by all increases in dietary protein, and decreased by all increases in non-protein nutrients.

Among the diets in which the supplements contained protein, the apparent digestibility of the nitrogen varied in the order of the variation in their protein contents, in part at least as results of the decrease in the proportion of the protein eliminated as metabolic products in the feces.

The partition of the energy of the diets is represented by table 3.

The digestibility of all the dietary components was so high that there could be little difference in the digestibility of the energy of the entire diets, the extreme difference in the average daily fecal energy in the fourteen experimental periods being between 7,206 cal. and 8,529 cal. In six among the seven dietary comparisons the amount of the fecal energy from the supplemented diet was slightly greater than that from the basal diet of the preceding experimental period.

The outgo of energy in the urine was slightly but definitely higher from the diets containing the non-protein supplements, and much higher from the diets in which the supplements contained protein, than from the preceding basal diets.

In computing the metabolizable energy values of the diets a correction was made, as usual, for the non-metabolizable energy of the protein gained. This correction has been made in the caloric values of the urine as given in table 3.

The average metabolizable energy values of the diets were phenomenally consistent, the coefficients of variation of the twelve individual determinations on the seven basal diets being between 1.0% and 2.6% and, of the seven supplemented diets, between 0.8% and 1.4%.

By inspection of the seven average metabolizable energy values for the same basal diet, it will be observed that the extreme range of variation was between 30,149 cal. and 30,659 cal. in 40,090 cal. of gross energy; and a statistical study of the differences in the seven average determinations of the metabolizable energy value of the basal diet

TABLE 3

Partition of daily food energy.

RAT NUMBERS	FEEDING TREATMENTS	INTAKE			FECES	URINE [1]	METABOLIZABLE ENERGY HEAT PRODUCTION				ENERGY BALANCE
		Basal	Supplement	Total				Coefficient of variation		Coefficient of variation	
		cal.	*cal.*	*cal.*	*cal.*	*cal.*	*cal.*	%	*cal.*	%	*cal.*
1–12	Basal diet	40,090		40,090	7,460	2,092	30,538	1.2	23,977	5.7	6,561
1–12	Basal + cerelose	40,090	13,712	53,802	7,552	2,332	43,918	.6	26,753	4.6	17,165
13–24	Basal diet	40,090		40,090	7,627	2,218	30,245	2.6	24,262	9.5	5,983
13–24	Basal + beef protein	40,090	16,913	57,003	8,529	5,412	43,062	1.2	29,731	10.4	13,331
25–36	Basal diet	40,090		40,090	7,409	2,074	30,607	1.0	22,208	7.6	8,399
25–36	Basal + lard	40,090	14,186	54,276	7,819	2,225	44,232	1.0	24,474	5.4	19,758
37–48	Basal diet	40,090		40,090	7,824	2,117	30,149	2.5	24,195	7.6	5,954
37–48	Basal + cerelose + beef protein	40,090	15,577	55,667	7,712	4,039	43,916	.8	27,899	5.8	16,017
49–60	Basal diet	40,090		40,090	7,504	2,071	30,515	1.3	25,097	3.8	5,418
49–60	Basal + cerelose + lard	40,090	13,697	53,787	8,040	2,233	43,514	1.4	26,733	3.4	16,781
61–72	Basal diet	40,090		40,090	7,206	2,225	30,659	1.0	25,656	9.2	5,003
61–72	Basal + beef protein + lard	40,090	16,275	56,365	7,705	3,924	44,736	.8	27,495	5.5	17,241
73–84	Basal diet	40,090		40,090	7,229	2,257	30,604	1.1	23,945	6.6	6,659
73–84	Basal + cerelose +beef protein + lard	40,090	15,425	55,515	7,755	3,377	44,383	1.0	26,740	7.5	17,643

[1] Corrected for the non-metabolizable energy of the protein gained.

revealed that among twenty-one possible comparisons only one appeared to be significant (odds 37 to 1).

The coefficients of variability among the measurements of the heat production were much less satisfactory. For the seven basal diets they varied between 3.8% and 9.5%, and for the seven supplemented diets between 3.4% and 10.4%.

These variations presumably resulted from differences in animal individuality; in effects of environmental conditions such as heat, light, air pressure and noise; and in the status of the subjects as to whether awake or asleep. The only way to avoid embarrassing variability among individual measurements of the heat production in experiments with animals seems to be to work with single subjects on each experimental treatment — as many experimenters have done. Heat production by animals is a highly individual and labile function.

The values for energy balance in the fourteen experimental periods should be understood as resultants of 8-day measurements of food, urine and fecal energy computed to a 24-hour basis, and measurements of the heat production during relatively small parts of 2 experimental days raised to the 24-hour basis.

The derivation of the heat increments from cerelose, beef protein and lard, singly and variously combined, as determined in each case by difference between the heat production for a basal period and for the immediately following supplemented period, is indicated by the data in table 4, and graphically in figure 1.

In this figure the numerical values above the seven graphs represent approximately equally metabolizable, daily, gross energy intake of small calories in supplements fed in addition to the basal diet. The plus and minus values for protein, carbohydrate, and fat represent calories of increased or decreased heat production from these nutrients as results of the consumption of the nutritive supplements, the base of comparison in each case (represented by the horizontal line across the graph) being the amount of heat produced from the protein, carbohydrate or fat of the basal diet.

For the purpose of this presentation the energy of fat synthesis is considered to be derived from the katabolism of carbohydrate.

The feeding of beef protein, cerelose and lard, individually, as supplements to a complete basal diet, had the effect in each case to increase the production of heat from the kind of nutriment represented by the supplement, these dynamic effects diminishing in the order in which the nutrients are mentioned. Cerelose spared twice as much heat production from fat as from protein; beef protein spared about the same

TABLE 4

Derivation of heat production from cerelose, beef protein and lard, singly and variously combined.

RAT NUMBERS	FEEDING TREATMENTS	HEAT PRODUCTION	HEAT PRODUCTION FROM			
			Protein	Carbohydrate	Fat	Fat synthesis
		cal.	*cal.*	*cal.*	*cal.*	*cal.*
1–12	Basal diet + cerelose	26,753	4,884	21,538	0	331
1–12	Basal diet	23,977	5,974	15,725	2,272	6
1–12	Cerelose (by difference)	2,776	— 1,090	5,813	— 2,272	325
13–24	Basal diet + beef protein	29,731	14,599	14,270	661	201
13–24	Basal diet	24,262	5,259	16,425	2,569	9
13–24	Beef protein (by difference)	5,469	9,340	— 2,155	— 1,908	192
25–36	Basal diet + lard	24,474	4,014	15,004	5,456	0
25–36	Basal diet	22,208	6 762	14,813	584	49
25–36	Lard (by difference)	2,266	— 2,748	191	4,872	— 49
37–48	Basal diet + cerelose + beef protein	27,899	9,184	18,403	46	266
37–48	Basal diet	24,195	5,510	15,163	3,509	13
37–48	Cerelose + beef protein (by difference)	3,704	3,674	3,240	— 3,463	253
49–60	Basal diet + cerelose + lard	26,733	4,884	21,435	350	64
49–60	Basal diet	25,097	6,263	15,771	3,063	0
49–60	Cerelose + lard (by difference)	1,636	— 1,379	5,664	— 2,713	64
61–72	Basal diet + beef protein + lard	27,495	10,084	17,026	348	37
61–72	Basal diet	25,656	5,886	17,515	2,250	5
61–72	Beef protein + lard (by difference)	1,839	4,198	— 489	— 1,902	32
73–84	Basal diet + cerelose + beef protein + lard	26,740	8,540	18,069	0	131
73–84	Basal diet	23,945	4,995	17,190	1,755	5
73–84	Cerelose + beef protein + lard (by difference)	2,795	3,545	879	— 1,755	126

amounts of heat production from carbohydrate and fat; lard was highly effective in sparing heat production from protein, leaving the amount of heat produced from carbohydrate essentially unchanged.

The mixed supplements of cerelose and beef protein, and of cerelose, beef protein and lard, increased the production of heat from both protein and carbohydrate; the supplement of cerelose and lard increased the heat production from carbohydrate alone, and the supplement of beef protein and lard increased the production of heat from protein alone. All four mixed supplements, whether they contained

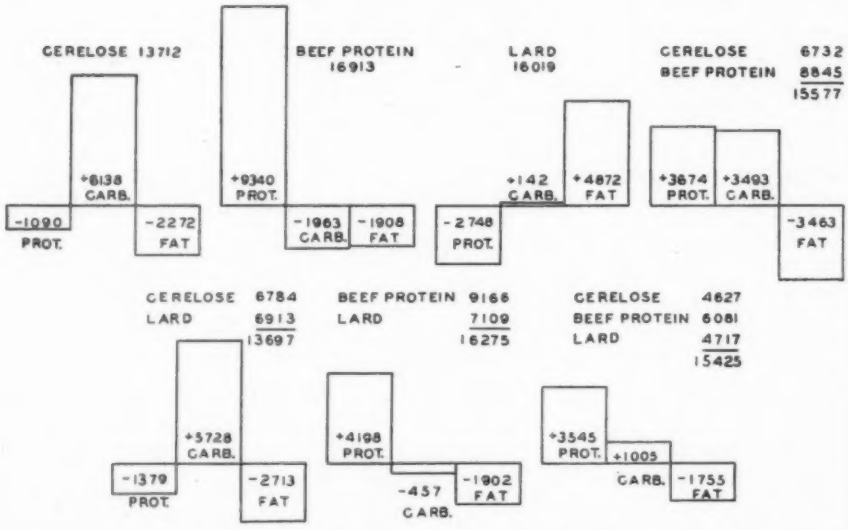

Fig. 1 The source of the dynamic effects of nutrients as affected by nutrient combination. This graph is explained in the text.

lard or not, decreased the production of heat from fat, thus emphasizing the energy reserve function of fat. The high dynamic effect of beef protein fed as a single supplement to a basal diet is not characteristic of beef protein fed as a component of a mixed supplement with cerelose or lard, or with cerelose and lard.

The energy values of the supplementary nutrients, individually and variously combined, are given in table 5. The metabolizable energy value of beef protein was found to be characteristically low, in spite of its high digestibility, because of the elimination of incompletely oxidized nitrogen compounds in the urine. Among the mixed supplements the metabolizable energy values of the three which contained

TABLE 5

Energy values of nutrients, individually and variously combined.

SUPPLEMENTARY NUTRIENTS	METABOLIZABLE ENERGY AS PER CENT OF GROSS		HEAT INCREMENTS AS PER CENT OF GROSS		HEAT INCREMENTS AS PER CENT OF METABOLIZABLE		NET ENERGY OF SUPPLEMENTS	NET ENERGY AS PER CENT OF GROSS	NET ENERGY AS PER CENT OF METABOLIZABLE	
	Experimentally determined	Computed from values for components	Experimentally determined	Computed from values for components	Experimentally determined	Computed from values for components			Experimentally determined	Computed from values for components
	%	%	%	%	%	%	*Cal./gm.*	%	%	%
Cerelose (3.42 Cal. per gram)	97.6	...	20.2	...	20.7	...	2.65	77.3	79.3	...
Beef protein (4.86 Cal. per gram)	75.8	...	32.3	...	42.7	...	2.08	43.5	57.3	...
Lard (9.46 Cal. per gram)	96.0	...	16.0	...	16.6	...	7.57	80.1	83.4	...
Cerelose + beef protein	88.4	85.9	23.8	27.2	26.9	30.8	2.62	64.6	73.1	66.4
Cerelose + lard	94.9	97.7	11.9	18.3	12.6	19.2	4.15	83.0	87.4	83.7
Beef protein + lard	86.5	82.2	11.3	24.4	13.1	28.2	4.74	75.2	86.9	66.9
Cerelose + beef protein + lard	89.3	86.7	18.1	23.1	20.3	25.9	3.59	71.2	79.7	71.2

protein were slightly higher as directly determined than as computed from the individual values for the components. The heat increments, or dynamic effects, of the nutritive supplements, as per cent of the gross energy, differed from the heat increments as per cent of the metabolizable energy mainly as influenced by the relatively low metabolizable energy value of the beef protein — the cerelose and the lard being almost completely metabolizable. The heat increment values of the mixed supplements, related either to the gross energy or the

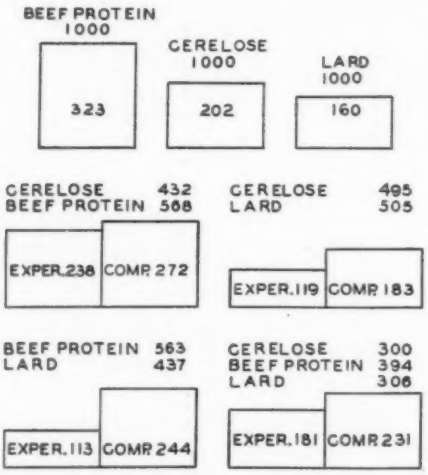

Fig. 2 The dynamic effects per thousand calories of gross energy of nutrients as affected by nutrient combination. This graph is explained in the text.

metabolizable energy, were in all cases materially lower as experimentally determined than as computed from the values for the individual components of these supplements, thus showing that nutrient combination favors efficiency in the utilization of metabolizable energy.

The heat increments, or dynamic effects, per 1000 cal. of gross energy of the individual nutritive supplements, and similar values for the four mixed supplements as directly determined and as computed from the values for their individual components, are graphically presented in figure 2.

The values above the rectangles represent in each case 1000 cal. of gross energy intake of supplementary nutrients, and the values inside the rectangles represent the dynamic effects of these nutritive supplements.

The values for dynamic effects of mixed supplements expressed as "Exper. . . ." were as experimentally determined, while those expressed as "Comp. . . ." were computed from the separately determined dynamic effects of beef protein, cerelose and lard; the amounts by which the experimentally determined values were lower than the computed values expressing the extent to which nutrient combination effected economy of utilization.

In the separate determinations of the dynamic effects of supplementary beef protein, cerelose and lard it was observed that the dynamic effect of the protein was 32%, of the carbohydrate 20%, and of the fat 16%, of their respective gross energy or fuel values; and such individual values are generally but erroneously considered to apply to normal diets in which these nutrients occur variously combined. Upon such individual values, commonly determined at planes of nutrition below energy equilibrium, depends the prevalent misconception that the dynamic effects of diets differ in accord with their protein contents.

The most efficient utilization of 1,000 cal. of food energy of the mixed supplements characterized the mixture of beef protein and lard. The dynamic effect of this combination was lower even than that of the supplement of lard alone (113 cal. compared with 160 cal. per 1000). The least efficient utilization of 1000 cal. of food energy of the mixed supplements characterized the one of cerelose and beef protein (238 cal. per 1,000). The dynamic effects of the combinations of cerelose and lard, and of cerelose, beef protein and lard, were intermediate between the extremes noted.

The well-known effect of fat to delay digestion does not account for the low heat increments of diets containing fat because these increments were determined not from single feedings of the test nutrient but by difference between observations on the heat production from established states of nutrition.

In offering numerical expressions of the associative effects of nutrient combination on the economy of utilization of food energy one can say that the experimentally determined dynamic effect of carbohydrate and protein was 12.5% less than as computed from the dynamic effects of the individual nutrients; and similarly, the dynamic effect of carbohydrate and fat was 35% less, of protein and fat 54% less, and of carbohydrate, protein and fat 22% less, than as computed from the dynamic effects of the individual nutrients.

Referring again to table 5, the net energy values of the mixed supplements, as per cent of their metabolizable energy, are shown to be materially higher, especially for those containing protein, as experi-

mentally determined than as computed from the individually determined net energy values for the protein, carbohydrate and fat — thus expressing the final effects of nutritive combination on energy value.

The dynamic effects of diets, therefore, are not the additive effects of their components, and are not in accord with their protein contents; and inasmuch as there is no scientific means of apportioning energy effects or values among dietary constituents, the dynamic effects of individual foods or nutrients are without significance as constants.

While there is a question as to the applicability of results obtained with one species of animal to the nutrition of another species, the results of this study suggest, at face value, that it is not necessary to diminish the protein content of the hot-weather human diet in order to have a low heat increment, since this purpose can be accomplished by the equicaloric substitution of fat for carbohydrate.

It thus appears that there is physiological economy in the high fat content of the diet of working people of some tropical countries — notably Brazil.

That the protein contents of diets do not dominate the heat increment is shown by five series of experiments conducted at this laboratory (Forbes et al., '35, '38, '39a, '40), with mature as well as with growing rats as subjects, in which the heat production of animals receiving equicaloric diets differing in protein content decreased slightly in the order of the increase in protein.

Also the results of this study imply that manufacturing processes which decrease the fat content of by-product feeds serve to lower the net energy value of the products not only through diminishing their gross energy but also by increasing the energy expense of their utilization.

SUMMARY

A study was made, with mature albino rats as subjects, of the dynamic effects of beef protein, cerelose (corn sugar) and lard, individually and variously combined, at planes of nutrition above maintenance; the dynamic effects being measured by difference between the heat production of the subjects as established on a complete basal diet and on the same plus the nutritive supplements.

The supplementary feeding of protein, of carbohydrate or of fat increased the production of heat from the kind of nutriment fed. Cerelose spared protein and fat; beef protein spared carbohydrate and fat; while lard spared only protein.

Of the mixed supplements, cerelose and beef protein increased heat production from carbohydrate and protein, and spared fat; cerelose and lard increased heat production from carbohydrate, and spared protein and fat; beef protein and lard increased heat production from protein, and spared carbohydrate and fat; while cerelose, beef protein and lard increased heat production from protein and carbohydrate, and spared fat. All mixed supplements, regardless of composition, spared fat.

Fed as supplements to a complete diet sufficient for maintenance, the dynamic effect of beef protein was 32%, of cerelose 20%, and of lard 16% of its gross energy.

The dynamic effects of mixed supplements of protein and fat, and of carbohydrate and fat, were lower even than the dynamic effect of fat. In this sense fat is much more potent than are protein and carbohydrate in determining the dynamic effects of diets.

Among four supplemental combinations of protein, carbohydrate and fat, computed to 1000 cal. of gross energy, the one with the lowest dynamic effect was that of protein and fat, followed closely by the combination of carbohydrate and fat; then by the combination of protein, carbohydrate and fat; while the combination with the highest dynamic effect was that of carbohydrate and protein.

The observed dynamic effect of carbohydrate and protein was 12.5% less, of carbohydrate and fat 35% less, of protein and fat 54% less, and of carbohydrate, protein and fat 22% less, than as computed from experimentally determined values for the individual nutrients.

The dynamic effects of diets are not the additive dynamic effects of their components; the prevalent idea that the dynamic effects of diets vary in the order of their protein contents is incorrect; and inasmuch as there is no scientific means of apportioning energy effects or values among dietary constituents, the dynamic effects of individual foods or nutrients are without significance as constants.

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THE BIOASSAY OF VITAMIN E[1]

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ONE FIGURE

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In an attempt to standardize the technique employed in the bioassay of vitamin E and thereby increase the accuracy of the test, a number of refinements have been made in the original method. Bacharach and co-workers ('37, '38) recommended the use of virgin animals. They found that rats in which sterility had been established showed more failures in implantation in the next pregnancy than did virgin animals. Bacharach ('38) also observed that a larger dose of vitamin E was required to insure fertility after a resorption gestation than with virgin rats.

Mason and Bryan ('38, '40) overcame initial storage by the feeding of a vitamin E low diet to the breeders during the latter half of the lactation period. The suckling young were thus limited in their vitamin E storage to that conferred through placental and mammary transfer. The weanlings were maintained on the vitamin E low diet; such prepared females invariably resorbed during their first gestation, thus eliminating the necessity of a trial pregnancy.

Mason ('39) studied the relation between the time of dosage and the response evoked. The best results were obtained if the test preparation was given on the eighth day of the gestation; however, if the test material was administered in several doses from the fourth to the eighth day inclusive of the pregnancy, the sensitivity of the test was further increased. Mason ('42) later reported that the state of the uterus on the sixteenth day of pregnancy was the best index of vitamin E activity. The presence of two or more viable fetuses in the uterus on the sixteenth day of pregnancy was considered a positive response.

[1] Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. The following materials were generously contributed: natural alpha-tocopherol by Merck and Company, Inc., Rahway, New Jersey; alpha-tocopheryl acetate by the League of Nations Committee on Vitamin Standardization; yeast by the Vitamin Food Company, New York; and calciferol by the Withrop Chemical Company, Rensselaer, New York.

This shortened assay period not only effected a saving of time and materials but contributed appreciably to the accuracy of the test. This technique, however, is subject to criticism in that resorption of the fetuses may be initiated late in the gestation.

Palmer ('37) evaluated vitamin E activity by reference to the percentage of total implantations resulting in living or in dead young. A live litter efficiency of 10% or more was considered a positive response.

Gottlieb, Quackenbush and Steenbock ('43) found that within certain limits females maintained on a low fat vitamin E free diet increased in weight during pregnancy in direct proportion to the quantity of vitamin E administered. For an equal response, the requirement for vitamin E on the low fat diet was less than half of that on the high fat ration. Homrich ('43) has recently reported that the use of a fat-free basal diet during the dosing period resulted in higher live litters and live implantation efficiencies than were observed with the standard lard, cod liver oil containing ration.

The studies herein reported were undertaken in an effort to increase the sensitivity of the vitamin E assay.

METHOD AND RESULTS

Virgin and resorption gestation rats were employed. The test materials were administered in single or multiple doses. Two types of diets were fed, one in which the vitamin E was destroyed by the incipient rancidity of the lard in the ration, and the other in which the vitamin E was removed by rigorous extraction of potential vitamin E containing ingredients in the diet with fat solvents. The diets used are given in table 1.

Littermate sisters were segregated at weaning so that each of the dietary groups was represented by one littermate. The rats on such dietary regimens were employed for three studies as follows: growth (the rats in this study were not bred), resorption gestation (the animals in this group were bred for a trial gestation and were used for curative assay in a subsequent pregnancy), virgin (the animals in this category were employed for cures after sterility had been established in the resorption gestation group).

Growth

The early growth rate of rats maintained on diet 427 was somewhat superior to that obtained with the other diets (fig. 1). This perhaps

TABLE 1

Diets deficient in vitamin E.

Diet 427 is made deficient by incipient rancidity of the lard. In the diets 821 A–D the vitamin E is removed by extraction with fat solvents.

DIETARY COMPONENT	DIET	
	427	821 A–D
	gm./100 gm.	*gm./100 gm.*
Casein		
(Tech.)	27	..
(Alcohol ether extracted)	..	24
Cooked ground corn starch	35	..
Sucrose	..	72
Salts (McCollum 185)	4	4
Brewers' yeast	10	..
Lard	22	..
Cod liver oil	2	..

Supplements fed daily to groups receiving diets 821 A–D.

	821A	*821B*	*821 C*	*821D*
Carotene (S.M.A.)	80 μg.	80 μg.	80 μg.	..
Cod liver oil	..	..	..	40 mg.
Calciferol	12 IU	12 IU	12 IU	..
Ethyl linoleate	60 mg.	..	..	..
Peanut oil[1]	..	250 mg.	250 mg.	250 mg.
Brewers' yeast	..	..	1 gm.	1 gm.
Ether extracted brewers' yeast	1 gm.	1 gm.	..	..

[1] 250 mg. of the oil was equivalent in its linoleic acid content to 60 mg. of ethyl linoleate. The peanut oil employed in these studies failed to invoke fertility in rats of proved sterility, when fed to eight rats at a level of 8 gm. (2 gm. on alternate days during the first 7 days of the gestation).

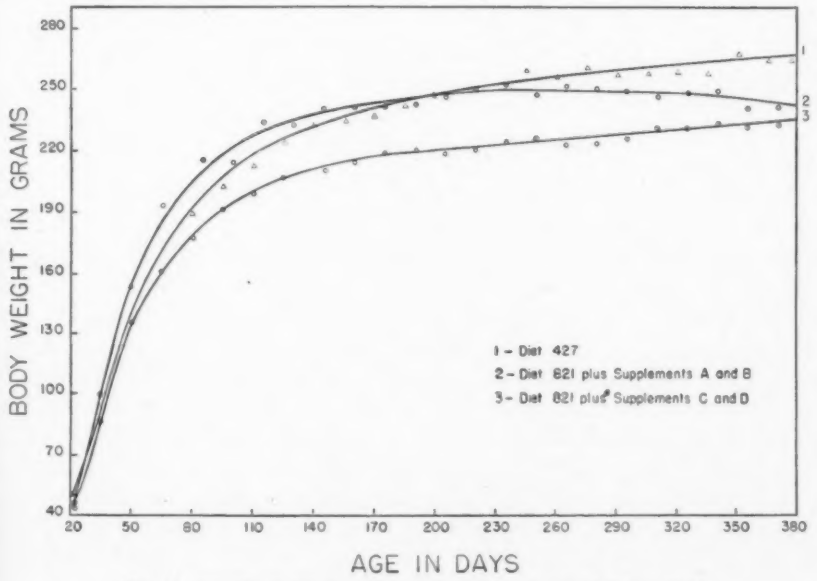

Fig. 1 Growth of female rats on the various types of E-low diets.

can be explained either by the potential growth stimulation of the fat in the ration or by the fact that the yeast and cod liver oil were incorporated in the diet while supplements were offered to the animals in the remaining groups. The rats did not consume the vitamins in their entirety for a period of a fortnight or longer. Slightly better growth was observed with rats receiving the unextracted than the extracted yeast.

First litter fertility

Eight to ten rats in the several dietary groups were bred for their trial gestation at the first pro-oestrus after 60 days. These rats were mated in late summer, a time of year in which first litter fertility was known to occur. As can be seen from table 2, the highest incidence of first litter fertility was observed with the rats receiving the lard, cod liver oil containing ration (diet 427). These rats resorbed in a subsequent gestation.

TABLE 2

Incidence of first litter fertility in rats reared on vitamin E-low diets.

DIET	NUMBER OF RATS	NUMBER OF RESORPTIONS	NUMBER OF LITTERS	AVERAGE NUMBER OF YOUNG PER LITTER	AVERAGE WEIGHT IN GRAMS
427	10	2	8[1]	7.8[2]	6.1
821A	9	8	1	1.0 (dead)	5.0
821B	10	8	2	3.5	5.0
821C	8	7	1	8.0	5.8
821D	9	7	2	4.0	4.6

[1] Two were questionable; loss of weight indicated that small litters were cast.
[2] Average of 6.

Curative assays with single doses

A single dose of a 3 mg. of alpha-tocopherol was administered on the first day of the gestation. Resorptions (table 3) resulted only with those rats receiving the cod liver oil or lard and cod liver oil in their rations (see diets 427 and 821D). The response to the single dose of the tocopherol was essentially the same for the virgin and for the resorption gestation groups. It is of interest to note that in late life, muscular dystrophy was observed only in rats receiving diet 427 despite the fact that the first litter fertility of early adulthood indicated a greater storage of vitamin E than in the other dietary groups.

As judged by litter quality,[2] the young cast by mothers receiving diets without cod liver oil were slightly superior to those of the

[2] The number of young per litter, their average weight in grams, the percentage of dead young in the litter, and the appearance of the live young.

mothers maintained on the cod liver oil containing rations; therefore, the use of carotene and calciferol is indicated.

Virgin rats and rats of proved sterility served equally well as test animals. In our opinion, the latter are preferable as the need for controls is obviated.

TABLE 3

The vitamin E activity of a single dose of 3 mg. of alpha-tocopherol administered to virgin and resorption gestation rats maintained on vitamin E-low diets.

DIET AND GROUP	NO. OF RATS	NO. OF IMPLANTATIONS	NO. OF RESORPTIONS	% OF IMPLANTATIONS LITTERING	NO. OF LITTERS WITH LVG. YOUNG	AV. NO. OF LVG. YOUNG PER LITTER	AV. WT. IN GMS.	LITTERS OF DEAD YOUNG
427								
Virgin	10	10	1	90	6	5.6	4.6	3
Resorption	7	7	1	86	5	6.8	4.3	1
Total	17	17	2	88	11	6.1	4.5	4
821A								
Virgin	10	10	0	100	10	6.6	5.2	0
Resorption	8	8	0	100	8	7.1	5.4	0
Total	18	18	0	100	18	6.8	5.3	0
821B								
Virgin	10	10	0	100	9	7.7	5.5	1
Resorption	9	9	0	100	9	7.7	5.7	0
Total	19	19	0	100	18	7.7	5.6	1
821C								
Virgin	10	10	0	100	10	6.7	5.5	0
Resorption	7	7	0	100	7	8.1	5.9	0
Total	17	17	0	100	17	7.3	5.8	0
821D								
Virgin	10	9	1	89	8	6.6	5.7	0
Resorption	8	8	1	88	7	6.6	5.5	0
Total	18	17	2	88	15	6.6	5.6	0

Curative assays — divided and single doses — diet 427

A comparison was made of the response to multiple dosing with several levels of alpha-tocopheryl acetate of virgin and resorption gestation rats maintained on the standard diet 427. Positive controls received 3 mg. of tocopherol as a single dose or in divided dosage.

The alpha-tocopheryl acetate fed in these experiments was supplied by the League of Nations Committee on Vitamin Standardization. The

TABLE 4

The vitamin E activity of several levels of alpha-tocopheryl acetate with virgin and resorption gestation rats maintained on diet 427.

GROUP	NO. OF RATS	NO. OF IMPLANTATIONS	NO. OF RESORPTIONS	% OF IMPLANTATIONS LITTERING	NO. OF LITTERS WITH LVG. YOUNG	AV. NO. OF LVG. YOUNG PER LITTER	AV. WT. IN GMS.	LITTERS OF DEAD YOUNG
Divided dose 0.8 mg. alpha-toco. Ac.								
Virgin	20	20	17	15	1	8.0	4.0	2
Resorption	13	12	10	17	2	3.3	3.3	..
Total	33	32	27	16	3	4.8	3.7	2
1.2 mg. alpha-toco. Ac.								
Virgin	19	19	9	53	10	4.0	4.3	..
Resorption	12	12	5	59	7	4.6	4.1	..
Total	31	31	14	55	17	4.3	4.2	..
1.8 mg. alpha-toco. Ac.								
Virgin	20	20	2	90	17	7.4	4.9	1
Resorption	13	13	1 *	92	9	6.1	4.5	3
Total	33	33	3	91	26	7.0	4.8	4
2.7 mg. alpha-toco. Ac.								
Virgin	20	20	0	100	20	8.6	5.2	..
Resorption	13	13	1 *	92	7	8.3	5.0	5
Total	33	33	1	97	27	8.5	5.2	5
3.0 mg. alpha-toco.								
Virgin	10	9	0	100	9	8.7	5.7	..
Resorption	11	11	0	100	11	7.9	5.8	..
Total	21	20	0	100	20	8.3	5.8	..
Single dose 3.0 mg. alpha-toco.								
Virgin	10	10	2	80	8	8.9	6.0	..
Resorption	15	14	1	93	11	7.0	5.7	2
Total	25	24	3	88	19	7.8	5.8	2

* Late.

test solutions were diluted with olive oil so that the total dose for one rat was contained in 0.5 ml. The single dose contained in 0.5 ml. of olive oil was fed on the first day of the gestation (as detected by the presence of sperm in the vaginal canal), the divided dose as 0.1 ml. on each of the first 5 days of the gestation. Negative controls received only the solvent; positive controls 3 mg. of natural alpha-tocopherol. The animals destined to receive each sample were selected over a 1- or 2-day period; thus each dilution was used for 5 or 6 days. The solutions were kept at 2°C. and saturated with CO_2 when not in use.

Early resorption gestations occurred in the negative control group indicating that rats maintained on diet 427 from day 21 and starting a gestation at 110 days were sterile although first litter fertility may ensue if the gestation is initiated at 60 days.

Significant differences in the percentages littering in the virgin as compared with the resorption gestation animals were not observed, but the litter quality was somewhat superior in the virgin group. The results for the virgin and the resorption gestation groups are tabulated both separately and combined (table 4).

The minimal effective dose to insure fertility appeared to lie between 1.8 and 2.7 mg. of alpha-tocopheryl acetate. The divided dose had a slight advantage over the single dose.

DISCUSSION

The low fat cod liver oil free rations were superior to the standard high fat diet. The incidence of first litter fertility was materially decreased by the feeding of the low fat ration; the administration of a single dose of 3 mg. of alpha-tocopherol produced a somewhat higher litter response with rats receiving the extracted diet than with the high fat rations. The superiority of the low fat type of diet is in agreement with the findings of Gottlieb et al. ('43) and Homrich ('43).

The virgin rat afforded little advantage over the animal of proved fertility. The failures in implantation reported by Bacharach and co-workers ('37, '38) were encountered only occasionally in either the virgin or the resorption gestation groups.

The response of a group of animals maintained on the standard diet (427) to a single dose of alpha-tocopheryl acetate indicated that the most sensitive response was obtained when the test material was administered over a period of several days. Litter quality paralleled the percentage littering.

SUMMARY

A comparison was made of the standard lard cod liver oil containing diet with rations composed of vitamin E free ingredients.

During the period of early growth the rats receiving the high fat ration grew at a more rapid rate than did their sisters maintained on the low fat diets, but after several months significant differences were not apparent. The rats receiving a diet supplemented with brewers' yeast attained a slightly higher weight plateau than did the rats receiving extracted brewers' yeast.

The highest incidence of first litter fertility was observed with rats maintained on the high fat diet; nevertheless, the rats maintained on this ration were less sensitive as test animals for vitamin E assays than were rats raised on the extracted diets. Unextracted yeast can be employed to advantage in vitamin E low rations. Peanut oil may replace ethyl linoleate as a vitamin E free source of the essential fatty acids.

Multiple, as contrasted with single dosage of alpha-tocopherol, appeared to result in a higher percentage of litters.

Virgin and resorption gestation rats served equally well as test animals. First litter fertility was encountered if the trial gestations were initiated at 60 days, but not at 110 days.

The minimal effective level of alpha-tocopheryl acetate to insure fertility in rats maintained on the high fat standard ration is between 1.8 and 2.7 mg.

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CONGENITAL MALFORMATIONS INDUCED IN RATS BY MATERNAL NUTRITIONAL DEFICIENCY

VI. THE PREVENTIVE FACTOR [1]

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Congenital malformations have been described in the offspring of female rats reared and bred on a diet of the following percentage composition: yellow corn meal 76, wheat gluten 20, calcium carbonate (C.P.) 3 and sodium chloride (C.P.) 1. This diet supplemented every tenth day by 60 I.U. of vitamin D as viosterol was called diet I. The deformed offspring showed shortening of the tibia, mandible, fibula, radius and ulna, fusion of ribs, fingers, and toes, and cleft palate. While there were quantitative differences of the malformations in different specimens, they all conformed to a definite pattern, the "pattern of diet I" (Warkany and Nelson, '40, '41 and Warkany, Nelson and Schraffenberger, '43a, '43b). Identical malformations were obtained in the offspring of rats of the Sprague-Dawley strain (albino), Long-Evans strain (hooded) and a strain of albino and hooded rats obtained from the Pediatric Department of Johns Hopkins University. It became obvious that a nutritional phenomenon was being dealt with since females of the same strains did not produce offspring of the pattern of diet I when they were bred while receiving an adequate stock diet. It was also found that the addition of 2% pig liver to the maternal diet I prevented the appearance of these abnormalities. When females, which had produced abnormal offspring while on diet I, were given diet I supplemented with liver before they were mated again, they always produced anatomically normal young. This led to the conclusion that a dietary factor deficient in diet I and present in large amounts in pig liver was necessary for the normal prenatal development of the rat. The search for this preventive factor will be described in the following pages. An inquiry into a maternal nutritional deficiency which

[1] This work was aided in part by a grant from the Nutrition Foundation, Inc., New York, New York.

manifests itself in the offspring differs from other investigations concerned with the finding of a vitamin or a vitamin-like factor. The stores of the maternal tissues act as "buffers" which prevent deprivation of the developing embryo as long as possible. In fact, it was assumed until recently that these maternal stores either protect the offspring completely, thus resulting in the delivery of normal young, or that in case of extreme dietary deficiency the embryos die in utero. Although there is some truth in this "all or none" theory, it is not entirely correct. Between the two there exists a narrow range in which maternal nutritional deficiency may result in arrest of the embryos' development without causing death. In this case congenitally deformed offspring may be the result.

The breeding of animals fed the deficient diet I gave results which conformed to this concept. Certain difficulties became apparent in the search for the maternal dietary factor capable of preventing malformations in the offspring. The females reared and mated while fed diet I either had no issue at all, or they produced normal young, or they had — comparatively rarely — deformed offspring. Under the most favorable circumstances only one-third of the young were anatomically defective. Abnormal offspring were less often the result of first than of second and later pregnancies. Thus, in order to demonstrate the preventive power of a supplement to diet I, it was necessary to obtain a large number of normal young from females that had been bred repeatedly. We considered a supplement to be preventive when more than 300 young, the results of first, second and third litters were found anatomically normal on external inspection and after clearing with the Schultze-Dawson method. On the other hand, the appearance of a very few abnormal young on a supplemented diet provided strong evidence that the supplement was not preventive.

We reported in previous communications (Warkany and Nelson, '42a; Warkany, Nelson and Schraffenberger, '42) that supplements of liver and alcoholic liver extract were preventive, while supplements of iodine, manganese, casein, alfalfa leaf meal, and cod liver oil were not preventive. Since then we have found that a supplement of 4% of liver ash is not preventive. Wheat germ oil (14 drops weekly administered by pipette) as a supplement to diet I did not inhibit the appearance of abnormalities. Since liver contains many vitamins of the B-complex in large quantities, we proceeded to test several crystalline substances of this group.

Thirty-eight females were fed diet I supplemented by 5 mg. each of riboflavin, thiamine hydrochloride, niacin, calcium pantothenate and

pyridoxine hydrochloride per 100 gm. of the diet. From these 38 females 55 litters consisting of a total of 371 young were obtained. Two hundred and thirty-three offspring were the result of first, 101 of second, 19 of third and 18 of fourth pregnancies. All 371 young were anatomically normal. It appeared, therefore, that the combination of the 5 crystalline vitamins mentioned prevented the abnormalities of the pattern of diet I. Another group fed diet I supplemented by thiamine hydrochloride, niacin, calcium pantothenate, and pyridoxine hydrochloride in the same quantities, produced a number of abnormal offspring (table 1). A supplement of pyridoxine hydrochloride plus calcium pantothenate failed to prevent the abnormalities. Thiamine hydrochloride alone as well as niacin alone were insufficient supplements, while riboflavin alone

TABLE 1

The effect of vitamin supplementation of diet I on the appearance of congenital malformations.

MOTHERS FED DIET I SUPPLEMENTED BY:	OFFSPRING		
	Total	Normal	Abnormal
Riboflavin + thiamine + niacin + pyridoxine + Ca pantothenate	371	371	0
Thiamine + niacin + pyridoxine + Ca pantothenate	51	27	24
Pyridoxine + Ca pantothenate	67	47	20
Thiamine	152	125	27
Niacin	180	161	19
Riboflavin	319	319	0

proved preventive. In the group receiving diet I plus 5 mg. of riboflavin per 100 gm. of diet, 36 females produced 55 litters consisting of 319 anatomically normal offspring. Of these young 211 were the result of first, 100 of second, and 8 of third pregnancies. These experiments seemed to indicate that riboflavin is the preventive factor sought for.

The crucial experiment remained to be done, namely, the production and prevention of the malformations on highly purified diets in which most of the nutritional factors were of known chemical constitution and in which the vitamin B-complex was represented by crystalline preparations only. It has been briefly reported (Warkany and Schraffenberger, '43) that female rats on a purified diet lacking riboflavin produced a small number of young with malformations which were identical with those of the pattern of diet I. The diet used for this purpose had the following percentage composition: sucrose 68, vitamin

test casein 18, vegetable oil 10,[2] and salt mixture 4.[3] One hundred grams of this diet were supplemented by 0.8 mg. thiamine hydrochloride, 0.8 mg. pyridoxine hydrochloride, 1 mg. calcium pantothenate, 10 mg. niacinamide [4] and 100 mg. choline chloride.[5] Every tenth day 1 to 2 drops of a 1:4 dilution with olive oil of "Drisdol with Vitamin A in Oil" [6] were administered by pipette.

We have repeated these experiments and improved the yield of abnormal offspring by keeping the females on the riboflavin deficient diet for about 3 weeks before breeding. The diet described above was also somewhat modified in that it was supplemented every tenth day by vitamin E (1 drop of a solution of 5 gm. alpha tocopherol in 100 ml. of olive oil), vitamin K (1 drop of a solution of 1 mg. of 2-Methyl-Naphthoquinone in 5 ml. of olive oil) and vitamins A and D (2 drops of a 1:4 solution of "Drisdol with Vitamin A in Oil"). This supplemented purified diet without riboflavin will hereafter be called diet Z. The females, which were about 3 months old and weighed about 200 gm. were put on diet Z for 3 weeks after which, whenever they were in estrus, they were bred to males which had been kept on an adequate stock diet. Some of the females became pregnant immediately, others only after several matings and a third group became sterile before they were impregnated. Of 54 females of this group 25 have had litters up to the present time. There were 25 first and only one second litter. These litters consisted of 137 young of which 104 were anatomically normal and 33 were abnormal. The abnormalities were entirely identical with those of the pattern of diet I.

Thirty-four females were fed diet Z supplemented by 0.8 mg. riboflavin per 100 gm. of diet. Twenty-seven of these females had litters. There were 27 first, 2 second and 1 third litters. These litters consisted of 120 anatomically normal young (table 2).

These experiments with purified diets are in accord with the assumption that a deficiency of riboflavin in the maternal diet is responsible for the abnormalities of the pattern of diet I in a part of the offspring.

About ½ of the females fed diet Z for several weeks stopped having estrus cycles, lost considerable weight and showed signs of "malnutrition". An attempt was made to "restore" these animals by giving them small amounts of riboflavin by pipette. It has been our experience that rats are unable to reproduce on diet Z when their weight falls

[2] The mixture of sucrose, casein and vegetable oil was furnished by S.M.A. Corporation.
[3] Salt mixture no. 351 as recommended by Hubbell, Mendel and Wakeman ('37).
[4] Merck.
[5] SMACO.
[6] Winthrop.

to 175 gm. or below. To such animals we gave 4 drops of an aqueous solution of crystalline riboflavin (12 mg. of riboflavin in 100 ml. of distilled water). As a rule this treatment resulted in a gain of weight within 24 hours. After 2 or 3 days there was usually no further increase and there might be loss of weight. Then another 4 drops of the riboflavin solution was given. This process of "titration" was continued until the animals resumed estrus cycles and regained a weight of about 200 gm. Then these restored females were bred. Up to the present time litters have been obtained from 7 of these females. In 4 of them the addition of riboflavin was discontinued after they conceived, while 3 females received 4 to 16 drops of the riboflavin solution during pregnancy. From these restored females 7 litters consisting of 52 young were obtained. Twenty-eight of these young or 53.9%, were

TABLE 2

Breeding results on diet Z and on diet Z plus riboflavin.

MOTHERS FED	OFFSPRING		
	Total	Normal	Abnormal
Diet Z	137	104	33
Diet Z + riboflavin	120	120	0

abnormal. This, in our experience, is the highest incidence of abnormalities obtained in any group of rats bred on a deficient diet. The procedure of depletion and controlled restoration of riboflavin may represent the best method of obtaining female rats which possess just enough riboflavin for reproduction but not enough for the normal development of the young. However, not all depleted animals can be restored by riboflavin. After a period of more than 4 months on diet Z, attempts at restoration have failed.

COMMENT

Various authors have attributed congenital malformations to a maternal nutritional deficiency. Zilva, Golding, Drummond and Coward ('21) observed that sows fed a diet deficient in vitamin A produced pigs in which some limbs were completely missing. Hughes ('34) reported that a purebred Poland China gilt fed a diet deficient in vitamins A and G produced six pigs, three of which were deformed. Converse and Meigs ('31) observed congenital blindness in the offspring of cows fed overripe timothy hay which had a low vitamin A content. Moore and co-workers ('35) found congenital blindness in calves, caused

by constriction of the optic nerve. They attributed the disorder to a nutritional deficiency of the dams which had received rations with a poor quality roughage. Hale ('33) described pigs without eyeballs born of a Duroc-Jersey gilt which had received a ration deficient in vitamin A. This author deserves credit for demonstrating that the defect was not genetically determined. In later publications Hale ('35, '37) described harelip, cleft palate, accessory ears and misplaced kidneys in pigs born to sows fed a vitamin A deficient diet. Several reports deal with embryonic defects in fowl which can be attributed to maternal nutritional deficiency. Micromelia and shortness of the antero-posterior axis of the skull were found by Byerly and co-workers ('35) in embryos of hens fed a deficient diet. Addition of wheat germ, liver and whey to the maternal diet prevented the abnormalities. Lyons and Insko ('37) observed chondrodystrophy in the chick embryo produced by manganese deficiency in the diet of the hen. Lepkovsky and co-workers ('38) reported that riboflavin deficiency in the breeding hen's diet produced defects such as degeneration of the wolffian bodies and reduced size of the embryo associated with death.

The experiments reported in the present communication add further proof to the thesis that maternal dietary deficiency may lead to congenital defects in the offspring. Since the malformations of the pattern of diet I could also be induced by a highly purified maternal diet without riboflavin (diet Z) and prevented by the addition of riboflavin to diet I or diet Z, it is concluded that the abnormalities of this pattern are caused by a prenatal riboflavin deficiency. This conclusion is in agreement with the fact that diet I is poor and liver rich in riboflavin. It may seem surprising that a supplement of 2% of alfalfa leaf meal to diet I did not prevent the abnormalities, since alfalfa is considered a good source of riboflavin. However, the riboflavin content of alfalfa plants is reduced to a great extent by maturation and curing (Hunt and Bethke, '40) so that a supplement of 2% may be insufficient for a pregnant rat.

We are dealing in these experiments with a borderline deficiency. The mothers apparently have sufficient riboflavin for maintenance of the estrus cycles and for gestation. The fetuses have sufficient riboflavin for growth but not for differentiation. It can be postulated that abnormal offspring appear when the riboflavin of the blood reaches a certain critical level. A reduction of riboflavin below the critical level leads to sterility or embryonic death, while an increase beyond this level results in the birth of normal young. It is understandable that such a borderline deficiency may lead to the production of normal, abnormal and even mixed litters. Slight variations of the fetal growth

rate, of the implantation sites or of the riboflavin level may decide whether the development of the fetus takes a normal or an abnormal course. Even under strict dietary control, many difficulties are encountered in attempts to obtain abnormal young. Synthesis of riboflavin by intestinal microorganisms and coprophagy may influence the maternal riboflavin reserves. Furthermore the mothers often resorb abnormal young in utero or eat them after birth, if they are not removed immediately.

The abnormalities of the pattern of diet I are most conspicuous in the skeleton. The attempt has been made to explain the pathogenesis of these malformations on the basis of histologic (Warkany and Nelson, '42b) and experimental findings (Warkany, Nelson and Schraffenberger, '42). It can be assumed that the formation of the membranous skeleton, which precedes the cartilaginous as well as the osseous skeleton, is inhibited by the riboflavin deficiency. That a general nutritional deficiency affects certain parts of the skeleton while others are spared may not seem plausible at first. However, similar differential susceptibility of developing organisms to toxic agents is well known (Child, '41). Our experiments show that such a differential susceptibility exists also to nutritional deficiency. This differential behavior is not obvious in the cephalocaudal direction, since bones of the head as well as those of the axial and appendicular skeleton are affected. However, in the transverse plane we note a differential sensitiveness, since in general the skeletal structures near the mid-dorsal region are normal while the more ventro-distally situated ones are more often deformed.

Gross malformations of soft tissues have been observed occasionally in the abnormal young of the pattern of diet I. Encephalocele, "open eye" (a severe ocular deformity), gastroschisis, reduction in size of the urogenital papilla and spina bifida were seen in rare specimens. We have not felt justified in including these rare defects in the pattern of diet I. However, thorough studies of the soft tissues have not yet been made. Such studies may reveal that the formation of the skeleton is not the only developmental process adversely affected by prenatal riboflavin deficiency.

SUMMARY

1. The congenital malformations of the pattern of diet I are prevented when the maternal diet I is supplemented by riboflavin.

2. Supplements of thiamine hydrochloride, niacin, pyridoxine and calcium pantothenate are not preventive.

3. On a purified maternal diet in which the vitamin B-complex is represented by crystalline substances, malformations of the pattern of diet I appear in the offspring when riboflavin is omitted.

4. On the same diet supplemented by sufficient riboflavin no deformed offspring are observed.

5. It is concluded that a deficiency of the maternal diet in riboflavin is responsbile for the congenital malformations of the pattern of diet I.

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ABSENCE OF RAPID DETERIORATION IN MEN DOING HARD PHYSICAL WORK ON A RESTRICTED INTAKE OF VITAMINS OF THE B COMPLEX[1]

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It has been claimed that diets markedly deficient in vitamins of the B complex provoke serious physical and psychological deterioration in a few days of hard work (Johnson et al., '42). If true this would be of much importance. However, we believe there were serious faults in the experiment and observations. The present report covers experiments in which detailed objective data were obtained under rigidly controlled conditions. The dietary limitations and the intensity of work were similar to those applied in the work of Johnson et al. ('42), parallel control subjects were studied, the period of restriction was twice as long, and "before" and "after" control periods were included.

PROGRAM

The general plan was to make frequent precise observations and measurements before, during, and after a period of hard physical work involving a daily expenditure of 4,500 to 5,000 Cal. A constant diet was used but the intake of B vitamins was varied by daily vitamin capsules or placebos so that a period of 14 days of low intake was preceded by an intake approximating National Research Council recommendations and was followed by 5 days of supplementation at a moderately high level. The responses were measured, in terms of physiological, biochemical and psychological variables, in set conditions and fixed tasks

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involving endurance, anerobic work strength, work capacity, speed and coordination.

The basal diet was very low in B vitamins but adequate in calories, protein, minerals and vitamins A, C and D. A rigid daily schedule of physical work, tests, meals and rest was followed. The subjects were divided into a control group of three men who received vitamin supplements daily and a "deficient" group of five men who received only placebos for a period of 14 days. Following the deficient period there was a period of 5 days when both groups received supplements. In this way both individual and group controls were provided.

During a preliminary period of 21 days the men were carefully standardized and control measurements and observations were made. This was followed by 14 days of "deficiency" and then 5 days of "recovery." In the last 19 days the daily intake of 4,640 Cal. was not quite sufficient to maintain caloric balance in any of the men; there was an average total loss of 2 pounds (maximum 4 pounds) in body weight. Throughout, all men rigidly followed fixed daily routines. The principal energy expenditure was caused by set tasks on the motor-driven treadmill. In the hard work period there were four forced marches daily of 60 minutes each at 3.5 miles per hour on the treadmill set at a 10% rate of climb. Pulse rates were counted at set times during and after this work. A battery of psychomotor tests was applied during work on alternate days at the start and at the end of the treadmill work. At the end of the second hour of this "forced" marching the men ran for 90 seconds at 9.0 miles per hour and a grade of 8.6% ("anerobic work"). At the end of the main work every other day additional special tests were made, such as the treadmill version of the Fatigue Laboratory Test (Johnson, Brouha and Darling, '42) and the Army Air Forces Fitness Test as used by Lt. L. A. Larson, A.A.F. The latter comprises "sit-ups," "push-ups" and a 300-yard shuttle race. Arm vein blood was drawn every fourth day during rest before and at 8, 12 and 30 minutes after the anerobic work. Every other day each man filled out a questionnaire on his subjective state. Detailed clinical examinations were made repeatedly. These involved the use of special opthalmological, electrocardiographic and roentgenographic methods. Urine collections (24-hour) were made frequently. The subjects were men 19 to 30 years of age with no signs or symptoms of significant abnormalities. Under complete supervision they worked, slept and ate entirely within the confines of the laboratory. The experiment rooms were maintained at 77°F. and 50% relative humidity at all times. Standard gymnasium clothing was worn in all experiments.

METHODS

All clinical analyses were made in duplicate with frequent blanks, standards and controls. Blood lactate was estimated by the method of Friedemann, Cotonio and Shaffer as modified by Edwards ('38). Blood pyruvate was estimated by the method of Lu ('39) as modified by Friedemann and Haugen ('42, '43). Blood sugar was measured by the method of Folin and Wu ('20) with cadmium hydroxide as the precipitating agent. Hemoglobin was determined as oxyhemoglobin with a photoelectric colorimeter. The 24-hour urine was collected in amber glass bottles containing 5 ml. of glacial acetic acid and 3 ml. of toluol. The samples were stored at 3°C. while awaiting analysis. Thiamine was estimated by the thiochrome method of Hennessy and Cerecedo ('39) as modified by the Research Corporation Committee for the Determination of Thiamine ('41). Riboflavin in food was determined by the microbiological method of Snell and Strong ('39) and in urine by the fluorometric method of Conner and Straub ('41). Niacin in food was measured by the method of Snell and Wright ('41). The thiamine method as used here has been checked for foods against standard biological assays.

Grip and back strength were measured with standard dynamometers. These measurements, as well as the flicker fusion frequency, were made in the morning before the beginning of the treadmill work and in the afternoon after the last standard 60-minute hard work period. Manual speed and control were tested with a ball-and-pipe test, motor control by tracing a stylus through a winding path, and speed of small movements by the tapping test as used previously (Keys et al., '43). Another test made during actual work on the treadmill was a measurement of reaction time involving gross bodily movements and postural adjustments.

Diet

Four fixed menus were used in rotation for all subjects. They provided an average of 35% of calories as fat. Protein adequacy was ensured (85 gm. daily average) by the use of vitamin-free casein and gluten. Only flavoring amounts of chicken, veal or beef were allowed (20 gm. daily). All flour and cereals were low-vitamin preparations. "Cream" was prepared by diluting a special 70% butterfat cream. The principal food items were macaroni or polished rice (400 gm.), bread (270 gm.), jello (150 gm.), butter (90 gm.), jelly (150 gm.), "cream" (300 gm.), hard candy and sugar (60 gm.), cake, cookies and pie (200 gm.) and farina (140 gm.). Very small amounts of canned

grapefruit, grape juice, pears, peaches, cherries, apples, strawberries and string beans made up the balance except for seasonings and token amounts of fresh lettuce and celery. Supplements supplied to all men daily ensured adequacy of minerals and of vitamins A, C and D according to National Research Council recommendations.

Every day each subject swallowed capsules, the contents of which were unknown to them or to the observers. The capsules used for the control subjects in the "deficient" period, and for all subjects in the "recovery" period contained yeast concentrate and pure vitamins providing a daily total of 3.25 mg. of thiamine, 2 mg. of riboflavin, 15 mg. of niacin, 25.2 mg. of pyridoxine, and 0.5 mg. of calcium pantothenate. Both vitamin and placebo capsules were identically flavored and colored.

TABLE 1

Average total (food plus capsules) vitamin intakes in milligrams per 1,000 Cal., and, in brackets, milligram per day for the three control subjects and the five "deficient" subjects. Average total caloric intakes per day are also given. Note the average caloric expenditures were approximately equal to the intakes for the "Preliminary" and "Control" periods but were about 150 to 200 Cal. per day greater than the intakes in the "Deficient" and "Recovery" periods.

PERIOD	PRELIMINARY	CONTROL	DEFICIENT	RECOVERY
No. of days	15	6	14	5
Cal. intake	3,560	4,430	4,640	4,640
Thiamine (control)	0.44 (1.57)	0.85 (3.78)	0.86 (3.99)	0.86 (3.99)
Thiamine (deficient)	0.44 (1.57)	0.85 (3.78)	0.16 (0.74)	0.86 (3.99)
Riboflavin (control)	0.56 (2.00)	0.68 (3.00)	0.59 (2.74)	0.55 (2.55)
Riboflavin (deficient)	0.56 (2.00)	0.68 (3.00)	0.15 (0.70)	0.55 (2.55)
Niacin (control)	5.9 (20.9)	5.3 (23.8)	5.0 (23.2)	5.3 (24.6)
Niacin (deficient)	5.9 (20.9)	5.3 (23.8)	1.8 (8.3)	5.3 (24.6)

It should be noted that prior to the experiment all of the subjects had subsisted for some months in a civilian public service camp where the intake of B vitamins was suspected but not proved to be low. In order to standardize the body stores of these vitamins, a period of 21 days preceding the "deficient" period was devoted to standardization of both the diet and the test procedures. Effort was made to approximate the National Research Council recommendations for this period. The actual intakes minus the N.R.C. recommendations for these caloric levels averaged, in mg. per man per day, for these 21 days as follows: Thiamine $= +0.14$, riboflavin $= -0.72$, niacin $= +1.0$.

All meals were prepared, weighed out and eaten under the supervision of a dietitian. Identical meals were analyzed for vitamins and stored at $-25°$C. for repeat analysis. The total daily vitamin intakes are summarized in table 1.

RESULTS

Clinical and subjective reports

No clinical changes occurred in any of the men at any time. All of the subjects were somewhat stiff and sore for the first 4 or 5 days of the hard work; this quickly disappeared and was not more prominent in the deficient than in the control group. Throughout the entire work period all subjects were tired at the end of each day as would be expected from the severity of the work. However, all of the men showed some training improvement. These phenomena were equally prominent in both groups. No anorexia, constipation, excessive fatigue, unusual muscle soreness, weakness, photophobia, cheilosis, depression, anxiety, irritability or sleeplessness occurred. The spirit was excellent at all times. The absence of subjective alterations associated with the level of the vitamin intake was clearly shown in the questionnaires. No changes were seen in electrocardiographic, x-ray or ophthalmological examinations.

Urinary excretion

Average urinary excretions of thiamine and of riboflavin for the two groups are given in table 2. The 24-hour output of thiamine clearly reflected the supplementation in the control group and remained at a

TABLE 2

Average 24-hour urinary excretion of thiamine and of riboflavin as per cent of the total intake of these vitamins. Column headings give the number of days from the start of the experiment. Compare with table 1.

PERIOD	PRE-LIMINARY	CONTROL	DEFICIENT			RECOVERY
Vitamin	1–12	19–20	25–26	29–30	33–34	40–41
Thiamine (control)	14	16	17	17	17	24
Thiamine (deficient)	15	14	14	7	5	14
Riboflavin (control)	27	27	26	26	35	37
Riboflavin (deficient)	28	29	13	26	29	28

fairly constant fraction of the intake in that group until the supplement was stopped. The deficient group started at much the same level but showed a rapid decline in both the absolute and percentage thiamine excretions. The excretion of riboflavin was similar to that of thiamine in the control group. In the deficient group, however, the excretion as per cent of intake did not decline though the absolute riboflavin excretion quickly fell to a low level.

Since the daily vitamin intake was accurately determined, each 24-hour urinary excretion measurement constituted in itself a "satura-

tion'' test. In addition, however, an extra 1.0 mg. of thiamine and 1.0 mg. of riboflavin were administered for ''saturation'' tests on the last day of the ''deficient'' diet, May 20th. The result for thiamine was an average recovery of 17.3% in the urine of the control group and 3.3% in the ''deficient'' group. For riboflavin the recovery averaged 37% in the control group whereas it was only from 1 to 6% in the ''deficient'' group.

Results of ''fitness'' tests

The grand averages for fitness, strength and psychomotor tests are summarized in table 3. In most of the tests, notably the Fatigue Laboratory Fitness test, all men showed some training improvement for several weeks but there was no significant differentiation between the two groups of men in any of the tests. The detailed records of the individuals offer no exceptions; i.e., as in the clinical and all other observations not a single man showed any changes which could be linked to the vitamin intake.

TABLE 3

Grand averages for the principal fitness, strength and psychomotor tests. ''Preliminary Period'' is the average for the tests in the last 5 days just prior to the deficiency period. ''Deficiency Period'' is the average for the tests in the last 6 days of the deficiency period. ''Recovery Period'' is the average for tests in the last 2 days of the ''recovery'' with supplements for all.

TEST [1]	PRELIMINARY PERIOD		DEFICIENT PERIOD		RECOVERY PERIOD	
	Control	Deficient	Control	Deficient	Control	Deficient
1. Anerobic, 1	178	172	171	168	171	172
2. Anerobic, 2	138	135	124	113	123	111
3. Endurance	133	115	118	108	123	112
4. Capacity	72	66	87	86	88	87
5. A.A.F. test	50	42	52	47	...	...
6. Grip strength	50	57	48	60	49	59
7. Back strength	305	333	328	325	358	347
8. Coordination	54	44	47	42	41	40
9. Speed — accuracy	64	62	67	64	67	68
10. Motor speed	66	67	65	63	66	63
11. Reaction speed	48	49	43	46	46	46
12. Flicker F. F.	59	60	58	58	61	59

[1] Test item no. 1 = pulse rate, in beats per minute, in the first 15 seconds after anerobic work; item no. 2 = pulse rate during 60 to 75 seconds after anerobic work; item no. 3 = average pulse during the last 5 minutes of 60 minutes of forced marching on the treadmill; item no. 4 = Fatigue Laboratory Test score; item no. 5 = A.A.F. Test score; item no. 6 = grip strength in kilograms; item no. 7 = back strength in pounds; item no. 8 = number of errors in the pattern tracing test; item no. 9 = ball-pipe circuits completed; item no. 10 = number of alternate plate taps in the first 10 seconds of tapping; item no. 11 = average gross body-posture reaction time, in units of 1/120 second, during work on the treadmill; item no. 12 = maximum flicker fusion frequency in flickers per second. Note that better performance is indicated by low scores in tests 1, 2, 3, 8, and 11.

Blood lactate and pyruvate

None of the blood lactate or pyruvate results could be interpreted as showing any relation to the level of vitamin intake. Ordinarily the most significant values are those during rest and at 12 minutes after anerobic work. The grand averages for these are summarized in table 4.

TABLE 4

Grand average values for blood lactate and pyruvate, in milligram of the acids per 100 cc. of blood, at rest and at 12 minutes after standard anerobic work. "Prelim." and "Recov." as in table 3. Other Column headings refer to the number of days on the deficient regime.

ITEM	PRELIMINARY		4TH DAY		8TH DAY		13TH DAY		RECOVERY	
	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient
Pyruvate, rest	0.9	0.9	0.9	0.9	0.8	1.0	1.0	1.0	0.9	1.0
Pyruvate, 12 min.	3.1	4.1	3.2	3.9	3.1	4.0	3.0	4.1	3.1	4.2
Lactate, rest	11	9	10	8	6	7	8	5	7	6
Lactate, 12 min.	72	96	55	74	41	62	50	63	55	62

DISCUSSION

The absence of essential data in the report by Johnson et al. (op. cit.) prevents exact comparison with the present results. In their study the energy expenditure, caloric intake, proportion of calories as fat, and the vitamin intake were unknown. However, proceeding from their food lists and their impressions of the level of energy output all reasonable assumptions lead to the same general rough estimates: their diet supplied, per 1,000 Cal., something like 0.12 mg. of thiamine, at least 0.25 mg. of riboflavin and perhaps 1.5 mg. of niacin. There is no basis for even this rough comparison for the other B vitamins but any difference between the two diets must have been small. The thiamine excretion of our subjects was apparently greater than that of the Harvard group but the method used by the latter for the determination must be questioned. It was reported that their "thiamine" subjects, who received 2 mg. of pure thiamine daily in addition to that in the diet, excreted an average of 200 μg. per day. This only amounts to about 8% of the intake which is widely at variance with all other experience at this level of intake.

The subjective symptoms described by Johnson et al. ('42), are of the vague type commonly related by the neurotic patient and easily induced by suggestion. They resemble those reported by Jolliffe et al.

('39). The latter study has been effectively criticized by Wang and Yudkin ('40).

The principal evidence offered by Johnson et al. ('42), for deterioration on their restricted diet is contained in their graph on p. 591. From this it appears that a marked loss in physical fitness occurred in 2 days and that up to the time yeast was given there was only a trivial difference at most between the men who received thiamine supplements and those who did not. The most consistent difference between the two groups appeared when they were all on exactly the same regime (the yeast period). The absence of proper controls is unfortunate.

The most reasonable explanation for the results reported by Johnson et al. ('42), is provided by a consideration of the subjects and the conditions of their experiment. A group of sedentary laboratory workers, highly conditioned to expect marked effects, were suddenly put to unaccustomed severe physical work and were fed a peculiar and monotonous diet. Apparently they all suffered in differing degrees from the symptoms which have been widely publicized for thiamine deficiency, even though half the men were given thiamine supplements. One man developed "cheilosis of marked degree," though the authors themselves consider that there was no riboflavin deficiency. The physical "fitness" test used is one which is readily affected by motivation and by the stiff and sore muscles resulting from excessive unaccustomed exercise. We have also seen sudden marked improvement in the score in this test after some days of hard training.

In previous work in this laboratory we have studied the relation between physical "fitness" and performance to vitamin "supercharging" (Keys and Henschel, '42) and to restriction in thiamine and riboflavin intake (Keys, Henschel, Mickelsen and Brozek, '43; Keys, Henschel, Michelsen, Brozek and Crawford, '44). We believe that the relation between "fitness" and vitamin intake is much less close than is popularly supposed to be the case (Keys, '43; Henschel, '43). In the present study we have attempted to maintain the most rigid controls and to apply the most sensitive objective measurements to various aspects of physical "fitness." The results justify very definite conclusions as to the absence of important effects in young men on the present restriction in B vitamins during several weeks of very hard work. This does not mean that we consider the vitamin intakes used here to be adequate for more than limited periods of time.

SUMMARY

1. Eight normal young men were maintained on a rigidly controlled regime of diet, physical work and exhaustive tests for 40 days. Twenty-one days were devoted to standardization with an average intake of B vitamins approximating the National Research Council recommendations. For the next 14 days the basal diet was constant and provided an average, in milligrams per 1,000 Cal., of 0.16 of thiamine, 0.15 of riboflavin and 1.8 of niacin. During these 14 days of "restriction" all men received daily capsules. Five men received placebos; each of the other three men received abundant daily supplements of yeast concentrate and synthetic B vitamins. During the last 5 days the same basal diet was used but all eight men received these supplements.

2. None of the men knew the contents of the capsules ingested. None of the observers knew which men were receiving supplements and which only placebos. All food was directly analyzed for B vitamins as eaten.

3. The energy level was carefully standardized by a set regimen of fixed tasks, most of them on motor-driven treadmills. Balance was maintained at 3,560 Cal. daily for the first 15 days and at 4,430 Cal. for the next 6 days. Thereafter (19 days) the average intake was 4,640 Cal. and the energy expenditure about 4,800 Cal. daily.

4. Comprehensive clinical examinations, including electrocardiography and special ophthalmological details, were made at the start and at the end. A fixed schedule of repeated carefully standardized tests was maintained throughout. These included twelve objective tests covering endurance, anerobic work, speed, coordination and muscle strength. Blood lactate and pyruvate were repeatedly measured at rest and at fixed intervals after standard exhausting anerobic work. The urinary excretions of thiamine and of riboflavin were measured every few days. Psychological questionnaires were filled out by each man every other day.

5. All results were in conclusive agreement that the vitamin intake and limitation were without effect on all the functions measured. Of all the variables measured and observed only the vitamin excretion in the urine reflected the intake.

6. Claims that normal men undergo physical deterioration in a few days of hard work on diets similarly restricted in B vitamins are seriously questioned. The results reported previously are explained on the basis of factors independent of the vitamin intake, namely, faulty experiments and lack of objective measurements and proper controls.

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ADDENDUM

Dr. Keys has kindly shown us this paper in which he reports results diametrically opposite to those we obtained 2 years ago in a study which was similar in general plan, and he has offered us this opportunity to discuss the discrepancies. The completeness and thoroughness of Dr. Keys work leave no doubt about the reliability of his results. Therefore one of two conclusions must be drawn: (1) either our experiments contained erroneous observations or conclusions, or both; or (2) the experimental conditions were sufficiently different to account for the difference in results.

Dr. Keys' chief criticisms are that our subjects were (1) sedentary, (2) suggestible, (3) unaffected by thiamine, and (4) that the analytical methods were poor. These criticisms, although perhaps not fully answered in the published work, were nonetheless covered in the complete protocols of our experiments. 1. Our subjects, as is customary among the workers in our laboratory, were experienced in outdoor work and in field trips and the majority had just completed experiments involving vigorous physical training. Their physical deterioration on the diet was completely different from their experience before and since on comparable field trips. 2. In answer to the criticism that suggestion was responsible for the changes observed we can only say that none of us expected any positive results in this short time. Furthermore, the thiamine pills and placebos were dispensed exactly as in Dr. Keys' experiments without the subjects knowing their content. 3. In answer to the criticism that there was no significant difference between the thiamine and placebo groups, we must point out that symptoms in the two groups were markedly different and that these symptoms were wholly different from those observed before and more recently on field trips under strenuous conditions. 4. The criticism of our analytic method for thiamine may be answered directly. The method of Egaña and Meiklejohn has proven reliable in (a) measuring accurately thiamine added to urine, (b) giving low blanks and good check values and (c) agreeing well with biological methods in the analysis of yeast.

These answers to the criticisms coupled with the results Dr. Ivy obtained which were similar to ours lead us to believe that our observations were real and our conclusions valid in the main. It would seem therefore more profitable to look to the second of the two possibilities mentioned in our first paragraph and to explore differences in experimental conditions in the hopes of reconciling the results. The main differences were: 1. Dr. Keys' subjects lived in a temperate laboratory environment; ours were outdoors in winter. 2. His subjects exercised wholly on a treadmill; ours in various outdoor activities. 3. The dietary thiamine intake of our subjects was undoubtedly lower, as shown not only by the estimates from the diet, but also from the

more rapid drop in urinary excretion of thiamine and the lower levels reached. 4. In our experiments the change in diet was more abrupt. Other smaller differences may be important, e. g., the type of food used. The recent demonstration of the biosynthesis of thiamine by Holt and Najjar complicates the entire picture. In view of this it may be necessary to consider many factors other than the intake of preformed thiamine. It is obvious that further work is necessary to clarify the problem fully.

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DIETARY PROTEIN AND PHYSICAL FITNESS IN TEMPERATE AND HOT ENVIRONMENTS [1]

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Recommendations for the daily protein intake of men who are doing hard physical work have ranged from 50 to 165 gm. The low figure is that of Chittenden ('04) and the high figure that of Rubner ('02) with intermediate estimates coming from many other authors (Lusk, '31, pp. 448 and 449). The present figure given by the National Research Council ('43) of 70 gm. daily represents a compromise. Most previous suggestions have stemmed from observations on men working in a temperate environment. For men working in hot environments recommendations are commonly made that protein intake be restricted (Rubner, '02; Lusk, '31) on the ground that its high specific dynamic action imposes an unnecessary load on the heat dissipating mechanisms of the body. However, in actual practice many groups of men in this country who are accustomed to working in the heat prefer a diet containing liberal quantities of meat. Good examples are harvesters, miners and professional baseball players.

In view of the practical and theoretical importance of the problem a study has been made of subjects subsisting on three levels of protein intake. Particular attention was paid to the quantitative measurement of the subject's physical fitness for work in temperate and in hot environments.

METHODS

The general plan of the experiment was as follows: Three healthy young men were subjects. The experiment covered four successive periods of at least 6 weeks each in the winter and spring of 1942–1943.

[1] This work was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College.

[2] We are indebted to the First Service Command, U. S. Army, for the services of Cpl. Joseph Poulin, Technician Fifth Grade, Pfc. A. Razoyk and Pfc. Joseph Stachelek.

The diets were nutritionally complete but varied from period to period with respect to level of protein. Observations in each period fell into three distinct categories: (a) observations on diet and nutritional state, (b) observations on physical fitness for work in both temperate and hot environments, and (c) observations on metabolism.

Diet and nutritional state

The subjects ate in a college dining hall. Total caloric intake was not restricted. Control observations were made in October and November with the subjects on their customary normal diet. From November 30th to January 11th the diet was high in first-class proteins with corresponding reduction in carbohydrate. From January 12th to March 6th the diet was sharply restricted in first-class proteins with corresponding increase in carbohydrate. During this period the diet was supplemented with 10 gm. of Fleischman's dried brewers' yeast [3] (type B 90) twice a week. This was to avoid the possible ill effects of B-complex deficiency. After March 6th the subjects returned to their customary diet. Dietary intakes were calculated according to standard tables (Bridges and Mattice, '42). Specimens of urine were collected frequently over 24-hour periods for estimation of nitrogen excretion. Samples of blood were drawn periodically from the subjects before breakfast for estimation of plasma protein. Urinary nitrogen and plasma protein were estimated by the micro-Kjeldahl method of Ma and Zuazaga ('42). Fasting body weight was measured frequently.

Physical fitness

In each dietary period physical fitness for hard work was tested both under temperate and under simulated desert and tropical conditions. A fourth subject subsisting at all times on a normal diet was included in all physical fitness measurements as a control against the effects of training and acclimatization.

Temperate environment. The test of Johnson, Brouha and Darling ('42) was used to assess physical fitness at 75°F., 50% relative humidity, and 0 m.p.h. wind velocity. In this test of stamina and cardiovascular fitness the subject runs on a motor driven treadmill at 7 m.p.h. up an 8.6% grade either until he is exhausted or, if not exhausted, for 5 minutes. On the basis of duration of effort and the sum of three pulses during recovery a score in arbitrary units is calculated. A change in

[3] We wish to thank Standard Brands, Inc., for the gift of this yeast.

score of about 3 or 4 points is significant. Each subject was given two such tests in each dietary period.

Hot environments. Performance in the heat was tested by prolonged marches on a treadmill. Short bursts of exhausting activity were found not to be well suited for observing responses to heat. Moist, tropical conditions were simulated at 95°F., 85% relative humidity and 5 m.p.h. wind. The subjects spent 10 to 12 hours a week in the heated room. In all tests of physical fitness in the heat the grade was 2.5%, the speed 3.5 m.p.h. and the length of performance 1 hour and 20 minutes. The maximal length of marching in 1 day was 5 hours. Salt intake was adequate at all times. Water intake was constant for each subject from experiment to experiment and was at a level which had been found to satisfy thirst at all times during the preceding normal period. Fitness was judged from terminal pulse rate and rectal temperature, equal weight being placed on each of these factors.

Metabolic observations

The effects of dietary protein level on respiratory metabolism were studied by a separate set of experiments in each dietary period under temperate (75°F., 50% relative humidity, and 0 m.p.h. wind) and tropical (95°F., 85% relative humidity, and 5 m.p.h. wind) conditions. These experiments on respiratory metabolism were distinct from those designed to test physical fitness. For each set of observations the subject came to the laboratory in a fasting state and rested while lying down for at least ½ hour in the appropriate room. His respiratory exchange was measured while reclining, standing and marching (after reaching a steady state) on a motor-driven treadmill at 3.5 m.p.h. Expired gases were collected in a Tissot gasometer or Douglas bag, and oxygen and carbon dioxide were estimated with the Haldane machine (Haldane, '35; Peters and Van Slyke, '31). In order to make the physiological stresses on the heat dissipating mechanisms comparable, a grade of 8.6% was used in the temperate room and a grade of 2.5% in the hot humid room. Neither grade of work was sufficient to cause an appreciable rise in blood lactate. Nitrogen in the urine during the periods of reclining, standing and marching was determined as described above. After completing the morning march the subject ate a late breakfast followed by lunch at the usual time. These meals were appropriate in protein content to the particular dietary period and contained approximately ⅔ of the protein intake for the day. One and a half hour after lunch the subject lay down again in the same

room as in the morning and the exact procedure of the morning was repeated. The total respiratory exchange and non-protein R.Q. were calculated according to standard methods (Lusk, '31, pp. 64–69; Lusk, '24).

RESULTS AND CONCLUSIONS

For the sake of clarity the results will be presented and discussed under the following headings: (a) observations on diet and nutritional state, (b) observations on physical fitness and (c) observations on respiratory exchange.

Observations on diet and nutritional state

Table 1 presents data on dietary intake, body weight, urinary nitrogen excretion and plasma protein concentration. The figures on dietary intake are for particular days considered to be typical of the subject

TABLE 1

Dietary intake, urinary nitrogen excretion, plasma protein and body weight as affected by type of diet.

	TYPICAL DIETARY INTAKE (PER DAY)				MEAN BODY WEIGHT	MEAN URINARY NITROGEN	MEAN PLASMA PROTEIN
	Calories	Protein	Carbohydrate	Fat			
		gm.	*gm.*	*gm.*	*kg.*	*gm./day*	*gm./100 ml.*
Averages							
Normal before	3040	105	391	117	70.1	12.9	7.2
High protein	3970	149	333	226	72.0	18.5	7.4
Low protein	3250	76	431	136	70.5	9.5	7.2
Normal after	3350	108	386	153	71.8	13.5	7.1

for the entire period under consideration. It will be noted that the average protein intake during the low protein period was 76 gm. per day, a rate of intake which would not be considered "low" by many authors. However, in view of the two following facts it may justifiably be called a "low protein diet." First, our subjects averaged over 100 gm. per day during both normal periods. Hence 76 gm. per day is a low protein diet for them. Second, our subjects were consuming over 3,000 Cal. per day, and with this caloric intake it is very difficult to reduce protein intake much below 76 gm. per day without resorting to highly artificial diets. Several points should be noted. First, urinary nitrogen excretion was highest during the high protein period and lowest during the low protein period. Second, plasma protein tended to be highest during the high protein period although its changes were slight. Third, changes in weight were small but maximal weight was attained during the high protein period.

Observations on physical fitness

Temperate environments. The figures given in table 2 are physical fitness scores derived as described above under METHODS. Each score is an average of two determinations made in the latter half of the particular period. Three points should be noted. First, subjects G.P., J.S. and Control showed a steady improvement from the start of the observations through the low protein period. This we believe to be a training effect resulting from the high level of physical activity of the subjects. Similar results have been noted in many other instances in this laboratory (Johnson, Brouha and Darling, '42). Subject J.P. reached a training plateau before the experiment started. Second, there are minor drops of 3 to 4 points in the scores of all subjects except J.S. during the normal period after. During this period as well as the latter part of the low protein period an epidemic of the common cold infected all subjects except J.S. We attribute this small decrease in

TABLE 2

Physical fitness scores in a temperate environment.
(75°F., 50% relative humidity. Each figure an average of two. Scores are in arbitrary units.)

	NORMAL BEFORE	HIGH PROTEIN	LOW PROTEIN	NORMAL AFTER
J.P.	40	40	40	37
J.S.	54	67	74	76
G.P.	85	90	93	90
Average	60	66	69	68
Control	..	68	76	72

physical fitness to these colds. Third, there were no changes in physical fitness other than those noted above either in the experimental subjects or in Control. Hence we conclude that for periods of at least 6 weeks dietary protein may be maintained at any level between 75 and 150 gm. per day without affecting physical fitness as measured in temperate environments.

These results are borne out by a field nutrition study made by this laboratory on twenty-one subjects (Darling, Johnson and Pitts, '43). They also correspond with results of other authors. Chittenden ('04) obtained similar results but gave them a different interpretation. His soldier subjects reported that they tired less easily and had more stamina on the low protein diet than on the preceding normal diet. Also the professional college athletic director in charge of training the subjects reported great improvement in their physical condition toward the end of their training period (low protein diet) as compared

with the start (normal diet). Chittenden attributed both the subjective and objective improvement to the low protein diet. It seems more reasonable to interpret his results as we have ours. That is to say, a vigorous training program such as that to which his men were subjected is almost bound to lead to increased vigor and hardihood, and any adequate diet will enable this improvement to proceed. Our work supports and extends that of Bassett, Holt and Santos ('22) who, using approximately the same levels of protein intake as we have, found no effects on physical fitness of high or low protein diets lasting for 1 week; however, we lengthened the dietary periods to 6 weeks or more and have employed a much more precise measure of physical fitness.

TABLE 3

Pulse rates and rectal temperatures during walking in hot environments. (Means of 4 to 9 measurements on different days.)

	NORMAL BEFORE		HIGH PROTEIN		LOW PROTEIN		NORMAL AFTER	
SUBJECT	Pulse rate /min.	Rectal Temp. (°F.)	Pulse rate /min.	Rectal temp. (°F.)	Pulse rate /min.	Rectal temp. (°F.)	Pulse rate /min.	Rectal temp. (°F.)
			Hot moist environment (95°F., 85% humidity)					
GP	134	101.4	128	101.5	131	101.4	128	101.5
JP	138	102.3	134	101.6	156	102.6	162	102.8
JS	143	102.1	141	101.6	139	101.6	134	101.4
Average	138	101.9	134	101.6	142	101.8	141	101.9
Control	...	....	162	102.7	156	102.8	160	102.8
			Hot dry environment (110°F., 25% humidity)					
GP	127	101.2	116	100.8	114	100.2	110	100.4
JP	146	101.7	138	101.1	128	101.0	126	101.2
JS	130	101.3	122	100.9	118	100.5	118	100.2
Average	134	101.4	125	100.9	120	100.6	118	100.6
Control	...	....	148	101.4	132	101.3	152	101.9

Hot environments. The physical fitness of the subjects for intermittent work in hot environments can be deduced from the data in table 3. Therein are listed the means of pulse rates and rectal temperatures measured at comparable times in experiments performed as described above under METHODS. It will be worthwhile at this point to clarify two terms used in this paper: "undue stress upon the heat dissipating mechanisms" and "negligible increase in heat production." Both of these concepts involve a distinction between mathematically real but physiologically insignificant increments of body heat. Healthy young men fully trained and acclimatized can perform physical labor in the heat in a physiological steady state provided water supply is unlimited

and the degree of exercise is not too severe. The body's mechanisms for maintaining heat balance are so efficient that this same steady state can be maintained under many conditions even when an increase is made in the amount of heat to be dissipated, provided the increase is small. We consider stresses upon the mechanisms for dissipating heat to be insignificant and increases in heat production to be negligible when they do not measurably affect the ability of a subject to maintain a given steady state.

The data in table 3 lead to three main conclusions. First, all subjects showed an improvement in performance during the first two or three periods. We believe this to be the residual acclimatization process described by Robinson, Turrell, Belding and Horvath ('43). These authors distinguish an initial period of about 7 days in which acclimatization to hot environments is quite rapid followed by a long-continued period of much slower acclimatization. All of our subjects had completed the initial period of rapid acclimatization before this experiment began. Second, subjects J.P. and Control exhibited a decline in performance during the last one or two dietary periods. We attribute this to the colds which were previously mentioned as affecting physical fitness in temperate environments. Third, there were no changes in performance at either 95°F. and 85% humidity or 110°F. and 25% humidity which are attributable to dietary protein level. In other words dietary protein may be maintained at any level between 75 and 150 gm. per day for periods of at least 6 weeks without affecting performance in a physical test which may increase the pulse rate by 60 or more beats per minute and raise the rectal temperature by 3 or more degrees Fahrenheit. This finding does not support the recommendation commonly made (Lusk, '31, p. 456) that meat be avoided in hot weather. Similarly Stefansson ('35–36) does not agree with this recommendation since he observed no unpleasant symptoms resulting from an all meat diet during a New York summer.

It has been alleged that increasing the dietary protein level significantly increases the loss of water in sweat. Lusk ('31, p. 409) considers an experiment of Rubner's in which the heat production and avenues of heat loss were studied in a man performing 850 Cal. of work in one day at 20°C. When the subject ate a diet of meat, more of the extra heat production due to work was lost by increased activity of the sweat glands than when he ate cane sugar only. This led Lusk to the conclusion that "a high protein dietary is therefore contra-indicated in athletic contests, especially when the weather is hot and humid." In the present experiments marching under standard conditions of work

in the hot, moist environment led to almost the same hourly loss of sweat independently of dietary period. The averages of the three subjects per hour of marching were: high protein period, 1.19 liters (twenty-two experiments); low protein period, 1.27 liter (twelve experiments). These results are different from Rubner's, and the reasons are made clear by examining Rubner's data. In the first place, his diets were artificial, employing on the one hand cane sugar and on the other meat. In daily life, as in our experiments, diets usually range from 50 to 200 gm. of protein per day. In the second place, the extra loss of water due to protein was, in Rubner's experiment, only 0.6 liters a day. This is an insignificant stress to the body when one considers that a normal, healthy young man is able to work day after day losing 10 or more liters of sweat. Only under conditions when water is strictly limited would this extra loss of sweat due to dietary protein become important.

Observations on respiratory exchange

We shall discuss separately the metabolism while reclining, standing and walking as it was affected by three factors: first, long-term changes due to prolonged subsistence upon the various levels of dietary protein; second, changes during individual days caused by ingestion of food; third, changes due to environment independent of diet. The full data are presented in table 4, the individual values for the three subjects being averaged for the sake of simplicity and clarity. It should be stressed initially that these experiments were not planned to demonstrate the relative specific dynamic action of isocaloric amounts of foodstuffs, but were intended to simulate normal daily life. The subjects, therefore, ate to satisfy hunger, being restricted in the proportions but not the quantity of foodstuffs eaten. Thereby are brought out certain practical points which compensate for the increased variability characterizing data collected under these conditions.

Reclining metabolism. In the present experiment the difference in protein content between the high and low protein diets (which may probably be considered as extremes for normal life) was about 400 Cal. per day. We may assume that the specific dynamic action of this increment is about 100 Cal. which is distributed throughout the 24 hours and probably amounts to not more than 6 Cal. in any 1 hour. Therefore a man with a basal metabolic rate of 1,800 Cal. per day is unlikely ever to increase that rate by more than 5% in changing from a low protein diet to a high one. These expectations are borne out by the data in table 4A. The basal metabolic rate during the high protein period was

1 to 2 Cal./m²/hr. above that during the low protein period. This was 3 to 5% of the total basal rate, a negligible amount from the standpoint of heat dissipation. A second point is that meals whether high, low or normal in protein content led to about the same increase in reclining metabolism, between 8 and 13 Cal./m²/hr. Finally, table 4A shows no consistent effects of environment on total reclining metabolism.

TABLE 4

Metabolism as affected by type of diet, by individual meals and by environments. (Average of three subjects.)

CATEGORY OF INTEREST	HIGH PROTEIN DIET		LOW PROTEIN DIET		NORMAL DIET	
	Fasting	After 2 meals	Fasting	After 2 meals	Fasting	After 2 meals
(A) RECLINING						
Temperate conditions (75°F., 50% relative humidity)						
Metabolism (Cal./m²/hr.)	39	48	38	46	36	49
Non-protein R.Q.	0.77	0.84	0.80	0.88	0.76	0.87
Pulse (beats/min.)	57	62	57	61	54	64
Rectal temp. (°F.)	..	..	98.0	98.4	98.2	98.6
Moist hot conditions (95°F., 85% relative humidity)						
Metabolism (Cal./m²/hr.)	39	47	37	48	40	49
Non-protein R.Q.	0.83	0.89	0.82	0.87	0.84	0.91
Pulse (beats/min.)	67	72	63	70	61	71
Rectal temp. (°F.)	98.4	99.1	98.5	99.1	98.5	99.1
(B) STANDING						
Temperate conditions (75°F., 50% relative humidity)						
Metabolism (Cal./kg./hr.)	1.15	1.39	1.15	1.33	1.14	1.41
Non-protein R.Q.	0.82	0.87	0.84	0.87	0.81	0.86
Pulse (beats/min.)	74	83	76	79	74	74
Rectal temp. (°F.)	..	..	98.4	99.0	98.5	98.9
Moist hot conditions (95°F., 85% relative humidity)						
Metabolism (Cal./kg./hr.)	1.26	1.44	1.20	1.39	1.17	1.48
Non-protein R.Q.	0.88	0.85	0.80	0.87	0.78	0.89
Pulse (beats/min.)	90	91	94	93	84	94
Rectal temp. (°F).	98.9	99.3	99.1	99.7	99.0	99.5
(C) MARCHING						
Temperate conditions (75°F., 50% relative humidity), speed 3.5 m.p.h., grade 8.6%						
Metabolism (Cal./kg./hr.)	7.80	7.96	7.48	7.67	7.36	7.54
Non-protein R.Q.	0.86	0.90	0.89	0.93	0.87	0.92
Pulse (beats/min.)	125	125	125	123	122	119
Rectal temp. (°F.)	..	..	99.4	100.0	99.5	100.1
Moist hot conditions (95°F., 85% relative humidity), speed 3.5 m.p.h., grade 2.5%						
Metabolism (Cal./kg./hr.)	5.09	5.24	4.95	5.13	5.22	5.34
Non-protein R.Q.	0.86	0.88	0.87	0.91	0.83	0.91
Pulse (beats/min.)	124	127	124	121	122	120
Rectal temp. (°F.)	99.9	100.4	100.0	100.6	99.7	100.0

Standing metabolism. Table 4B shows the average standing metabolism for the three subjects. It is expressed in terms of Cal./kg. body weight/hr. since standing metabolism is probably more nearly proportional to body weight than to surface area. The practical conclusions to be drawn from the table are similar to those in the case of reclining. First, the difference between the fasting metabolic rates during the high protein and during the low protein period was small, not exceeding 5%. Second, the post-prandial rise in metabolism varied from 0.18 to 0.31 Cal./kg./hr., being lowest in the low protein period. Third, there was a consistent small effect of environment on total standing metabolism, which was always slightly higher in hot than in temperate environments, regardless of diet or of meals. The difference was at the most 7 Cal. per hour for a 70 kg. man.

Marching metabolism. It will be recalled that in order to impose comparable stresses upon the mechanisms for heat loss, a lower grade of work was employed in the hot, moist room, where it was equivalent in severity to marching at 3.5 m.p.h. on the level with a 40-pound pack, a standard U.S. Army pace. Consideration of table 4C leads to two chief conclusions. First, the overall diminution in metabolism during work in changing from a high protein to a low protein diet amounted at most to 0.3 Cal./kg./hr. This constituted a decrease in the marching metabolic rate of only about 4%. It cannot be said that one is taxing the heat dissipating mechanism of the body when one increases a moderate metabolic rate by 4%. This conclusion is borne out by the actual performance of our subjects. Under environmental conditions which were extremely severe they performed at least as well in the high protein period as they did in the low protein period. The second conclusion is that regardless of protein content of the meals the post-prandial rise in metabolism never exceeded 0.2 Cal./kg./hr. and constituted a negligible heat load according to our criteria.

Non-protein R.Q. Three consistent changes of theoretical interest were noted in non-protein R.Q. First, under both environmental conditions and in all three types of activity there was a post-prandial increase in the non-protein R.Q. of the same magnitude in all three dietary periods. Second, in the reclining state the non-protein R.Q. was higher under moist hot conditions than under temperate conditions. Third, during marching the non-protein R.Q. tended to be lower under moist hot conditions than under temperate conditions. When "partition of nutrients" was calculated by the usual methods, the "percentage of metabolism due to carbohydrate" showed the same changes as the non-protein R.Q.

GENERAL DISCUSSION

The results of the present experiments have some bearing on certain practical aspects of human nutrition. It would appear that for periods of at least 6 weeks dietary protein level may vary widely (from 75 to 150 gm. a day) without affecting physical fitness for intermittent work either under temperate or under hot conditions. Since a high protein diet is preferred by most workmen, it should not be prohibited on the basis of effects on physical fitness or heat dissipation. However, in the special case where water supply is limited, as in a lifeboat, a high protein diet is contra-indicated for two possible reasons. First, the increase in waste nitrogenous products demands extra water for their excretion. Second, the specific dynamic action of protein presumably demands a small increase in perspiration. Neither of these two factors is of any practical significance under ordinary circumstances.

SUMMARY

1. The effect of variation in the level of dietary protein upon the physical fitness and metabolism of three subjects was studied under both temperate and tropical conditions while reclining, standing and marching.

2. The urinary nitrogen excretion in grams per day averaged 18.5 during the high protein period, 9.5 during the low protein period and 12.9 and 13.5 during the normal periods before and after the experiment, respectively. There were minor changes in body weight with a maximum during the high protein period. The plasma protein level showed no significant changes.

3. Physical fitness under temperate conditions showed no changes attributable to dietary protein level.

4. Performance of work in both hot, dry and hot, moist environments showed no changes attributable to dietary protein level. In both cases, however, improvements due to training and acclimatization were observed.

5. Metabolism while reclining and while standing was not significantly different in the high and low protein periods. Metabolism while marching was slightly lower in the low protein period. However, as judged by actual performance in the heat this was a physiologically insignificant change.

6. It is concluded that even though protein does have a high specific dynamic action, the theoretical objections heretofore raised against a high protein diet in hot environments are unjustified under the condi-

tions of our observations. Protein intake may vary widely from 75 to 150 gm. daily without effect upon performance of intermittent work in the heat.

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STUDIES ON THE COMPARATIVE NUTRITIVE VALUE OF FATS

IV. THE NEGATIVE EFFECT OF DIFFERENT FATS ON FERTILITY AND LACTATION IN THE RAT [1]

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The dietary requirement necessary to promote lactation adequate for the growth of the new-born rat is more stringent than that required for the growth of older animals or for reproduction. Lactation thus provides a more exacting criterion for the evaluation of the adequacy of a diet than the usual growth method.

Sure ('24a, b) demonstrated that diets satisfactory for growth and reproduction are in some cases inadequate for lactation. A larger intake of the B complex is required for lactation (Sure, '27) and possibly there is a specific component called "B_x", essential for milk production (Sure, '41a). Para-amino benzoic acid was shown to be a component of this factor, a finding which has recently been confirmed (Sure, '43); it was also demonstrated that inositol has an injurious effect on lactation which is counteracted by para-amino benzoic acid. Diets containing 18% of casein as the protein were unsatisfactory unless supplemented with cystine (Sure, '41b). Sure found that neither butter fat, lard, hydrogenated cottonseed oil, olive oil nor wheat germ oil was able to promote satisfactory lactation on an otherwise inadequate diet.

The present tests are an extension of earlier investigations (Deuel et al., '44) where comparisons were made of the growth of young rats on diets of mineralized vitamin-fortified skimmed milk powder containing butter and on similar diets containing corn, cottonseed, olive, peanut or soybean oil, or a margarine in place of the butter.

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In the present study, the effectiveness of such diets in supporting pregnancy and lactation has been followed in rats maintained on these diets from the time of weaning (21 days of age).

EXPERIMENTAL

Three series of experiments consisting of 207 individual tests were carried out. The rats in the first series (series II in Deuel et al., '44a) were bred at 133 days of age after having received the different diets for 16 weeks; those in the second series (series III in the earlier report) were mated at 105 days of age after having received the various diets for 12 weeks. The third series represents some of the rats of our present second series which were rebred 4 weeks after the birth of their first litter. In all cases females were mated with males which had received the same diets. In the first two series, the litters were reduced to seven animals at 3 days of age. Because of the marked effect of the size of the litter on rate of growth, we have averaged the weights of those rats only as long as the litters were maintained at seven animals.

In series I the weights were recorded only at 3 days and 21 days of age; in series II the weights are reported at 3, 7, 14, and 21 days. The litters in series III were reduced to two female rats at 3 days. They were weighed at 3, 7, and 14 days of age at which time they were weaned.

No flavoring was added to the diets in series I. However, after the demonstration in other experiments that flavor plays an important role in food consumption (Deuel and Movitt, '44b), diacetyl [2] or butter flavor [3] was added to the diets employed in series II and III. Other experimental conditions were similar to those in the earlier tests.

A statistical evaluation of the mean weight is applied only to the 21-day weights in series II and to the weights at 14 days in the third series. This is because the average values given for the weights at the earlier periods and for part of the litters at 21 days in series I are based on the litter weight rather than on the weight of the individual rats.

RESULTS AND DISCUSSION

The results of the various experiments are summarized in table 1. On diets composed of mineralized skimmed milk powder, and a fat fortified with the fat-soluble vitamins essential for the rat, successful

[2] Obtained from Larkin and Co., Buffalo, New York.

[3] "B.F.A." obtained from Verley Products Corporation, 1621 Carroll Avenue, Chicago, Illinois.

TABLE 1

The average weight of rats at several periods before weaning when the mothers had been raised and were maintained on diets of skimmed milk powder and different fats.[1]

DIET[2]	FEMALES BRED	LITTERS		NO. OF RATS IN LITTER AT BIRTH	WEIGHT OF RATS[3]				ANIMALS DIED
		Born	Destroyed		3 days	7 days	14 days	21 days[4]	
Series I					gm.	gm.	gm.	gm.	
I	9	9	1	9.5	7.67			31.0(42)	1
II	7	6	0	9.8	7.60			29.6(42)	0
III	6	6	0	7.8	7.96			30.4(14)	2
IV	10	10	1	6.0	6.95			25.9(14)	8
V	4	4	0	10.2	8.49			38.6(28)	0
VI	9	8	0	8.6	7.37			27.3(21)	2
VII	7	7	0	7.3	7.07			32.7(42)	0
Series II									
Ia	14	14	0	9.6	7.41	13.4(77)	24.6(56)	35.8±0.6(42)	22
II	16	14	1	8.9	7.62	13.2(77)	22.8(70)	34.9±0.7(70)	1
III	15	14	1	9.7	8.13	12.8(84)	22.9(84)	34.9±0.5(84)	0
IVa	15	15	1	9.3	7.83	13.6(98)	22.0(98)	36.1±0.5(98)	0
V	12	11	2	7.6	7.53	11.8(49)	21.7(49)	33.0±0.9(42)	1
VI	15	15	1	7.8	7.53	12.4(63)	23.4(63)	35.7±0.5(63)	2
VII	15	14	1	7.6	7.47	12.6(63)	22.8(63)	35.8±0.6(56)	1
Series III									
Ia	6	6	0	7.3	8.19	14.8	31.6±1.1 (12)		0
II	8	8	0	8.8	7.92	14.1	28.7±1.2 (16)		0
III	10	10	0	9.2	7.93	12.7	25.7±1.2 (20)		0
IVa	10	8	0	6.3	8.56	13.6	27.1±1.1 (10)		0
V	5	3	0	5.8	7.19	9.5	20.8[5] (4)		0
VI	8	8	0	7.9	8.68	14.9	30.1±1.7 (16)		0
VII	6	5	0	8.0	9.60	15.0	31.0±0.9 (10)		0

The figures in parentheses represent the number of animals on which the averages are based.

[1] The diets had the following composition: Mineralized skimmed milk powder 70.6%, the added minerals per 100 lbs. being $MnSO_4 \cdot 7H_2O$, 3.455 gm., $CuSO_4 \cdot 5H_2O$, 3.340 gm., $FeC_6H_5O_7 \cdot 3H_2O$, 45.557 gm.; a butter fat, a margarine fat, or vegetable oils supplemented with vitamins and flavor as indicated below per 1000 gm. fat (in series II and III where a whole butter or a whole margarine was used, these were increased so that the same amount of fat was present) 29.4%. The supplements in the fat were: vitamin A — carotene (40% of total A) 8 mg., vitamin A concentrate 133 mg., vitamin D — viosterol 801 mg., vitamin E and tocopherol 10 mg., diacetyl or commercial butter flavor 4 mg.

[2] The diets containing the various fats are designated as follows: I, butter fat; Ia, whole butter; II, corn oil; III, cottonseed oil; IV, a margarine fat; IVa, a whole margarine; V, olive oil; VI, peanut oil; VII, soybean oil.

[3] The average weight at 3 days is the mean weight of all rats. The average values at 7, 14 and 21 days in series I and II are for rats only in those litters where there were seven rats. In series III, the weight at 7 and 14 days are for two in a litter.

[4] Including the standard error of the mean calculated as follows:

$\sqrt{\frac{\epsilon d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[5] Too few experiments for statistical evaluation.

pregnancy ensued irrespective of whether the fat added was a butter, a margarine, or corn, cottonseed, olive, peanut or soybean oil. That the various diets are adequate for fertility in both males and females is indicated by the fact that over 94% of the females cast litters. If fat per se is required for fertility and pregnancy, its requirement may be equally well satisfied by the various vegetable fats as by butter.

From the standpoint of lactation, as judged by the survival of the litters and the weight of the young at weaning time, the different fats were also equally effective. No statistical differences were found in series II or series III where the groups were sufficiently large for a statistical evaluation. Although such an analysis was applied only to the 21- and 14-day weights respectively in these two groups, it is evident from the inspection of the average weight at 3, 7, and 14 days that these values for the rats on the various diets are all within experimental error. A possible exception is the olive oil group in series III which would appear to be definitely lower than the other groups. On the other hand, the rats receiving the olive oil diet in series I have a considerably higher average weight than the rats on the diets containing the other fats. This seeming discrepancy may be because the number of rats in series III which received the olive oil is too small on which to base any conclusions (only two litters). It might also be due to the inferior grade of olive oil which was used in the third series as contrasted with the first and second series when a more satisfactory sample was available. In series II, values of this group are within the experimental error of the other groups.

With the exception of the animals receiving the olive oil diet, the weight of the rats in series III at 14 days was 20 to 30% higher than in the second series. This is probably to be ascribed to the fact that the litters were reduced to two rats in the former case although there is also the possibility that it may partly be due to the fact that the rats in the third series were from second litters which are usually somewhat larger than those from the first litter.

The reason for the large mortality in the group receiving the butter diet in series II is unknown. This was not observed in the first or third series; moreover, growth of the mothers which had been started on this diet at 21 days of age had previously been shown to be satisfactory (Deuel et al., '44a).

SUMMARY

No differences were found in the fertility of male or female rats which from 21 days of age had received diets of mineralized skimmed milk powder fortified with the necessary fat-soluble vitamins and vari-

ous fats irrespective of whether the lipid was butter, a margarine, corn, cottonseed, olive, peanut, or soybean oil. Moreover, such diets were equally efficient in promoting lactation as judged by the weights of the rats when weaned at 14 or 21 days of age.

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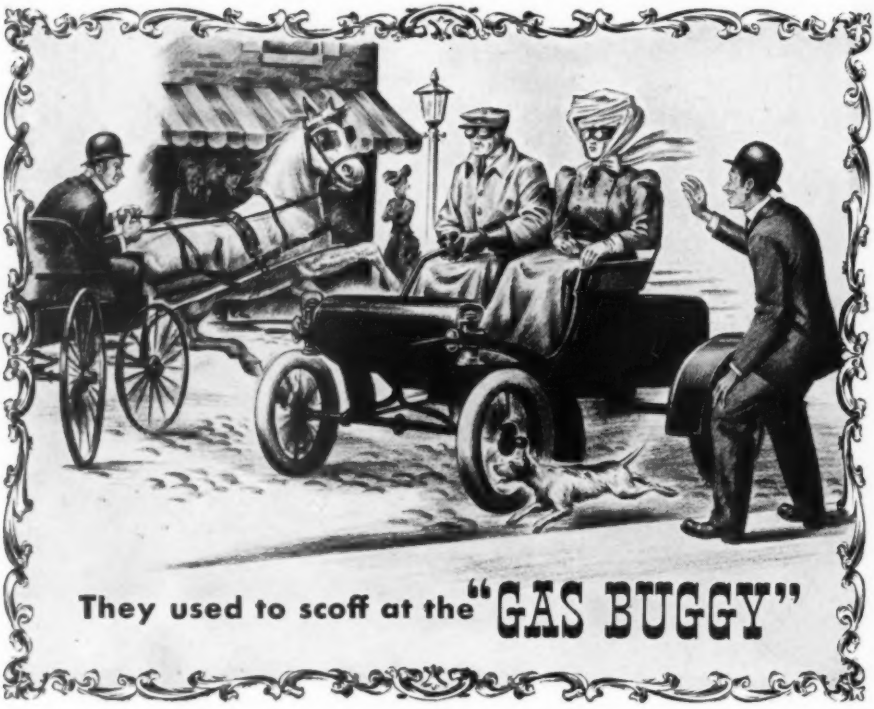

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1. The infant's initial store of iron is rapidly depleted during the first months of life. (Mackay,[1] Elvehjem[2]). About 30% of the iron freed from the hemoglobin during the first two months is lost, and while hemoglobin destruction takes place, all infants are in negative iron balance. (Jeans,[3] and Usher, et al.[4]).

2. During the early months of life the infant obtains very little iron from milk — 1.44 mg. per day from the average bottle formula of 20 ounces or possibly 1.7 mg. per day from 28 ounces of breast milk. (Holt,[5] Jeans[3]). The incidence of nutritional anemia has been found to be high among infants confined largely to a diet of cow's milk. (Davidson, et al.,[6] Usher, et al.,[4] Mackay[1]).

For these reasons and also because of the low hemoglobin values so frequent among pregnant and nursing mothers (Strauss,[7] and Gottlieb and Strean[8]), the pediatric trend is constantly toward the addition of iron-containing foods at an early age, both to normal infants and those with pylorospasm. (Neff,[9] Blatt,[10] Brennemann,[11] Monypenny[12]).

THE CHOICE OF THE IRON-CONTAINING FOOD

1. Many foods high in iron actually add very little to the diet because much of the mineral is lost in cooking or because the amount fed is necessarily small or because the food has a high percentage of water. Strained spinach, for instance, contains only 1 to 1.4 mg. of iron per 100 Gm. (Bridges[13]).

2. To be effective, food iron should be soluble. Some foods fairly high in total iron are low in soluble iron. Thus egg yolk and liver have less soluble iron than does farina, which is very low in total iron. (Summerfeldt[14]). Oxalate-containing leafy vegetables are low in soluble iron and appear not to be well utilized as a source of iron by infants. (Kohler, et al.,[15] and Stearns[16]).

3. **Pablum (and Pabena) are high both in total iron (30 mg. per 100 Gm.) and soluble iron (7.8 mg. per 100 Gm.) and can be fed in significant amounts at an early age, without digestive upsets. (Blatt,[10] Monypenny[12]). Clinical studies of sick and well babies have shown Pablum to be of value in raising hemoglobin values (Crimm, et al.,[17] Summerfeldt and Ross[18]), even when egg yolk and spinach were not effective (Stearns[16]).**

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1940—First isolation of solid Penicillin by Oxford investigators.

1940—Merck research on antibiotics concentrated on Penicillin.

1941—First report of Penicillin's clinical value.

1941—Prof. H. W. Florey and Dr. N. G. Heatley, of the Oxford group, visited the United States to confer with interested Government officials and manufacturers, with the objective of establishing Penicillin production in America.

1941—Dr. Heatley, who participated in the first production work in England, remained at the Merck Research Laboratories to collaborate with Merck chemists in developing test and production procedures.

1941—Merck brought about a reciprocal arrangement between British and American investigators to spur production in cooperation with the United States and British governments.

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